ANALYSIS OF ANTIULCER DRUGS WITH SPECIAL REFERENCE TO PROTON PUMP INHIBITORS IN PHARMACEUTICAL FORMULATIONS

ABSTRACT

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

CHEMISTRY

BY

ZEHRA BANO

DEPARTMENT OF CHEMISTRY
ALIGARH MUSLIM UNIVERSITY
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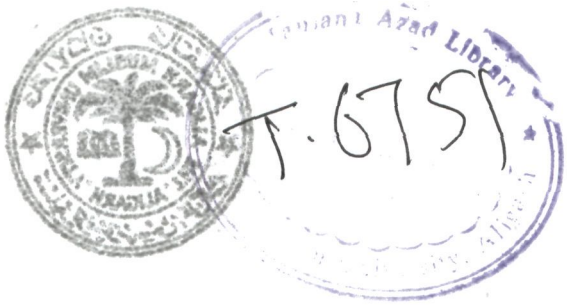

ABSTRACT

The thesis entitled **"Analysis of antiulcer drugs with special reference to proton pump inhibitors in pharmaceutical formulations"** is comprised of five chapters. The first chapter describes a general introduction of the subject matter. The very relevant matters include:

- a brief history of drug and its relation with our daily life
- importance of drug analysis and its role in testing the medicine before it is made available to the public
- a note on drug impurity profiling
- a brief description of various analytical techniques and its applications in the quantitation of drug in pharmaceuticals and biological fluids
- a description on process monitoring and validation
- a detailed information on statistical treatment of calibration data in order to facilitate the selection of parameters which are relevant to the performance and suitability of the given analytical procedure.

Once the analytical method is advent, it is necessary to decide its suitability for the intended purpose. This is known as the method validation. Now a days, a variety of strict demands are posed by numerous international organizations namely International Union of Pure and Applied Chemistry, International Laboratory Accreditation Conference, Western European Laboratory Accreditation Cooperation, International Conference

on Harmonisation, and International Organization for Standardization on such methods, the most important parameters for evaluation of method reliability and overall performances are:

- confirmation of identity
- solution stability
- selectivity/ specificity
- linearity
- accuracy and precision
- limits of detection and quantitation
- recovery
- robustness/ruggedness
- equivalence testing

A description of the classification of drugs based on pharmacological action on human organs is included and finally a brief literature and chemical structures of the four drugs, i.e., pantoprazole, lansoprazole, esomeprazole magnesium and rabeprazole sodium are presented. An abundant and well-composed list of references is given at the end of this chapter, comprises 228 citations, taken from the world's leading scientific journals in the field.

The second chapter describes a kinetic spectrophotometric method which is based on the oxidation of pantoprazole with Fe(III) in sulphuric acid medium. Fe(III) subsequently reduces to Fe(II), which is coupled with potassium ferricyanide to form Prussian blue. The reaction is followed spectrophotometrically by measuring the increase in absorbance with time

(1 - 8 min) at 725 nm. The initial rate method is adopted for constructing the calibration graph, which is linear in the concentration range of 5 - 90 μg ml^{-1}. The regression analysis yields the calibration equation:

$$v = 3.467 \times 10^{-6} + 4.356 \times 10^{-5} C.$$

The limits of detection and quantitation are 1.46 and 4.43 μg ml^{-1}, respectively. The proposed method was optimized and validated both statistically and through recovery studies. The experimental true bias of all samples is < ±2.0%. The method has been successfully applied to the determination of pantoprazole in pharmaceutical preparations.

In chapter three, a simple kinetic spectrophotometric method has been discussed for the determination of lansoprazole in pharmaceutical formulations. The method is based on the oxidation of the drug with alkaline potassium permanganate at room temperature. The reaction is followed spectrophotometrically by measuring increase in absorbance owing to the formation of MnO_4^{2-} at 610 nm (Method A) and decrease in absorbance at 530 nm due to disappearance of MnO_4^- (Method B). The calibration curves are linear over the concentration ranges of 5 - 150 and 5 - 70 μg ml^{-1} with the corresponding calibration equations:

$$\text{rate} = -3.915 \times 10^{-6} + 5.271 \times 10^{-5} C$$

and

$$\Delta A = 1.04 \times 10^{-3} + 1.78 \times 10^{-3} C$$

for methods A, and B, respectively. The statistical comparison of the results of the proposed procedures with those of the reference spectrophotometric method show excellent agreement and indicated no significant difference between the methods compared in terms of accuracy and precision. Interval hypothesis tests have also been performed which indicated that the true bias of all samples is less than ± 2%.

The fourth chapter describes two simple and sensitive spectrophotometric methods for the determination of esomeprazole magnesium in commercial dosage forms. Method A is based on the reaction of esomeprazole magnesium with 5-sulphosalicylic acid in methanol to form a yellow product, which absorbs maximally at 365 nm. Method B utilizes the reaction of esomeprazole magnesium with N-bromosuccinimide in acetone-chloroform medium to form α-bromo derivative of the drug peaking at 380 nm. Under the optimized experimental conditions, Beer's law is obeyed in the concentration ranges of 2 - 48 and 10 - 100 μg ml^{-1} with molar absorptivity of 2.11×10^4 and 4.57×10^4 l mol^{-1} cm^{-1} for methods A and B, respectively. The limits of detection for methods A and B are 0.35 and 0.46 μg ml^{-1}, respectively. No interference was observed from excipients commonly present in tablet formulations. Methods A and B are successfully applied to the commercial tablets for the estimation of esomeprazole magnesium with good accuracy and precision. The results are compared favorably with the reference spectrophotometric method indicating no significant difference between the methods compared.

The last chapter describes two selective and validated spectrophotometric methods have been described for the quantitation of rabeprazole sodium in commercial dosage forms. Method A is based on the reaction of drug with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) in the presence of ammonium cerium (IV) nitrate in acetic acid medium at room temperature to form red-brown product which absorbs maximally at 470 nm. Method B utilizes the reaction of rabeprazole sodium with 1-chloro-2, 4-dinitrobenzene (CDNB) in dimethyl sulphoxide (DMSO) at 45 ± 1°C to form yellow coloured Meisenheimer complex. The coloured complex has a characteristic band peaking at 420 nm. Under the optimized reaction conditions, proposed methods are validated as per ICH guidelines. Beer's law is obeyed in the concentration ranges of 14 - 140 and 7.5 - 165 μg ml^{-1} with linear regression equations:

$$A = 6.041 \times 10^{-4} + 1.07 \times 10^{-2} C$$

and

$$A = 1.020 \times 10^{-3} + 5.0 \times 10^{-3} C$$

for methods A and B, respectively. The limits of detection for methods A and B are 1.38 and 0.75 μg ml^{-1}, respectively. Both methods have been applied successfully for the quantitation of rabeprazole sodium in commercial dosage forms. The results are compared with the reference UV spectrophotometric method.

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DEDICATED
TO
MY PARENTS

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Certificate

This is to certify that the thesis entitled **"Analysis of antiulcer drugs with special reference to proton pump inhibitors in pharmaceutical formulations"** is the original work of Miss ZEHRA BANO, carried out under my supervision and suitable for submission for the award of the degree of Doctor of Philosophy in Chemistry.

Nafisur Rahman

(Dr. Nafisur Rahman)

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Zehra Bano

(ZEHRA BANO)

CONTENTS

LIST OF PUBLICATIONS

[1] Kinetic spectrophotometric analysis of pantoprazole in commercial dosage forms

Anal. Sci. 22 (2006) 983-988, The Japan Society for Analytical Chemistry.

[2] Kinetic spectrophotometric method for the determination of lansoprazole in pharmaceutical formulations

J. Serb. Chem. Soc. 2006 (in press).

[3] Spectrophotometric determination of esomeprazole magnesium in commercial tablets using 5-sulphosalicylic acid and N-bromosuccinimide

J AOAC Int. (2006) The Journal of AOAC International (communicated).

[4] Optimization and validation of spectrophotometric methods for the quantitation of rabeprazole sodium in commercial dosage forms

Chem. Pharm. Bull. (2006) The Pharmaceutical Society of Japan (communicated).

LIST OF TABLES

LIST OF FIGURES

CHAPTER-1

GENERAL INTRODUCTION

The discovery of new drugs and their development into commercial product takes place across the broad scope of the pharmaceutical industry. The basic underpinning for this effort is the cumulative body of scientific and biomedical information generated worldwide in research institutes, academic centers, and industries. The combined efforts of chemists, biologists, molecular biologists, pharmacologists, toxicologists and pharmaceutical scientists, engineers, and many others are involved in the drug discovery and development process.

The pharmaceutical industry in the United States grew rapidly during World War II and in the years immediately following. The upsurge in the domestic production of the drugs and pharmaceutical products stemmed in part from the wartime hazards and consequent undependability of overseas shipping, the unavailability of drugs from previous sources, and the increased need for drugs of all kinds, but especially those with life-saving capabilities. One such drug is penicillin, the antibiotic that became commercially available in 1944, 15 years after its discovery in England by Sir Alexander Fleming and one year before the end of the war.

New drugs may be discovered from a variety of natural sources or created synthetically in the laboratory. They may be discovered by accident or as the result of many years of tireless pursuit. Throughout history, plant materials have served as reservoir of potential new drugs. Yet, only a small portion of the approximate 270,000 known plants thus far have been investigated for medicinal activity. Certain major contributions to modern drug therapy may be attributed to the successful conversion of botanic folklore remedies into modern wonder drugs. The chemical reserpine, a tranquilizer and hypertensive agent, is an example of a medicinal chemical isolated by design from the folklore remedy *Rauwolfia serpentina.* Another

plant drug, periwinkle or *Vinca rosea,* was first scientifically investigated as a result of its reputation in folklore as an agent useful in the treatment of diabetes mellitus. Plant extactives from *Vinca rosea* yielded two potent drugs, which when screened for pharmacological activity surprisingly exhibited antitumor capabilities. These two materials, vinblastine and vincristine, have been used successfully in the treatment of certain type of cancer including acute leukemia, Hodgkin's disease, lymphocytic lymphoma, and other malignancies.

After the isolation and structural identification of active plant constituents, chemists may recreate them by total synthesis in the laboratory or more importantly use the natural chemical as the starting material in the creation of slightly different chemical structures through molecule manipulation procedures. The new structures, termed semisynthetic drugs, may have a slightly or vastly different pharmacological activity than the starting substance, depending on the nature and extent of chemical alteration. Other plant constituents that in themselves may be inactive or rather unimportant therapeutically may be chemically modified to yield important drugs with profound pharmacological activity. For example, the various species of Dioscorea popularly known as Mexican yams, are rich in the chemical steroid structure from which cortisone and estrogens are semisynthetically produced.

Animals have served humans in their search for drugs in a number of ways. They not only have yielded to drug testing and biological assay procedures but also have provided drugs that are mannered from their tissues or though their biological processes. Hormonal substances such as thyroid extract, insulin, and pituitary hormone obtained from the endocrine glands of cattle, sheep, and swine are life-saving drugs used daily as replacement therapy in the human body. The urine of pregnant mares is a rich source of estrogens. Knowledge of the structural

architecture of the individual hormonal substances has produced a variety of synthetic and semisynthetic compounds with hormone-like activity. The synthetic chemicals used as oral contraceptives are notable examples.

The use of animals in the production of various biological products, including serums, antitoxins, and vaccines has been of life-saving significance ever since the pioneering work of Dr. Edward Jenner on the smallpox vaccine in England. Today the poliomyelitis vaccine is prepared in culture of renal monkey tissue, the mumps and influenza vaccines in fluids of chick embryo, the rubella (German measles) vaccine in duck embryo, and the smallpox vaccine from the skin of bovine calves inoculated with vaccinia virus. New vaccines for diseases as AIDS and cancer are being developed through the use of cell and tissue cultures.

In recent years many new and important innovative therapeutic agents have been developed and approved by the Food and Drug Administration (USA), including drugs to treat: acquired immune deficiency syndrome (indinavir), refractory benign prostatic hyperplasia (sumatriptan), ovarian carcinoma (paclitaxel), gastric ulcers (lansoprazole, esomeprazole, rabeprazole, pantoprazole), hyperlipidemia (gemfibrozil), hypertension (enalapril), congestive heart failure (carvedalol), coronary artery disease (fluvastatin), obsessive compulsive disorders (fluoxetine), arthritis (nedocromil), ostioporosis (alendronate), male impotance (sindenafil), infectious disease (ciprofloxacin) and other diseases and conditions, with literally hundreds of potential therapeutic agents in various stages of clinical evaluation.

We live today in a world of drugs: drugs for pain, drugs for disease, drugs for allergies, drugs for pleasure, and drugs for mental health. Drugs that have been rationally designed; drugs that have been synthesized in the factory or purified from

nature have been clinically tested. It was supposed that the drugs act on the human organ effectively and safely. By no means was it always so. Before the end of the 19th century, medicines were concocted with a mixture of empiricism and prayer. Trial and error, inherited lore, or mystical theories were the basis of the world's pharmacopoeias. The technology of making drugs was crude at best: tinctures, poultices, soups, and teas were made with water or alcohol based extracts of freshly ground or dried herbs or animal products such as bone, fat, or even pearls, and sometimes from minerals best left in the ground - mercury among the favored. The difference between a poison and a medicine was a hazy differentiation at best: In the 16th century, Paracelsus declared that the only difference between a medicine and a poison was in the dose. All medicines were toxic. There is no known drug that is not harmful or even poisonous at high doses. In theory, a "goal drug" would produce the specifically desired effect, be administered by the most desired route at minimal dosage and dosing frequency, have optimal onset and duration of activity, exhibit no side effects, and following its desired effect would be eliminated from the body efficiently, completely, and without residual effect. The gradual change from the use of natural products in their entire state to either purified extracts from those products or to synthetic chemically - produced substances can be said to have been taking place between the time of Paracelsus, who lived in Basel during the first half of the sixteenth century, to that of Ehrlich, to whom the award of a Nobel prize in 1909 was a fitting reward for his remarkable researches and breakthrough during the first decade of this century. This period has been described as that leading from Quintessence to the Chemical and has been fascinatingly reviewed by H. J. Barber [1]. This transition from the Quintessence to the Chemical stimulated a very considerable amount of interest in the analysis as well as purity of natural products

to determine, as De Quincey [2] had said one hundred years earlier, not the apparent quantities as determined by weighing but the virtual quantities after allowing for the alloy of impurity. Thus, there is no doubt that nearly a century of pharmaceutical research has contributed spectacularly to improvement in human health and quality of life.

The growing awareness of the need to analyze drug substances was also apparent before any synthetic materials found regular using medicine. Higher standards for the preparation of pharmaceutical ingredients had been set, following the 1858 Medical Act's stipulation that the General Medical Council (U.K.) should produce a list of medicines and compounds and manner of preparing them together with true weights and measures by which they are to be prepared and mixed [3]. The first editions of British Pharmacopoeia and Japanese Pharmacopoeia were published in 1864 and 1886, respectively. Thereafter, pharmaceutical and fine chemical manufacturers laid greater emphasis on meeting the standards set by pharmacopoeias. In 1890, Jesse Boot established an analytical laboratory and its staff were involved mainly for analyzing proprietary medicines of competitors in order that Boots should develop new and / or cheaper formulations [4].

The increase in use of classical analysis to quantitate and define materials used in medicine, together with the increase in the use of materials of synthetic origin and of increasing complexity, is evident from a study of pharmacopoeias of various countries issued between 1900 and 1950. Shortly after this period one can see the exciting beginnings of a completely new era in the analysis of drugs based on the introduction of new concepts of analytical methodology that found no reference in the classical analytical textbooks of the time. The introduction of complexometric and non-aqueous titrimetry in the early 1950s, gas liquid chromatography in 1952,

thin layer chromatography in 1956 together with the increasing applications of ultra-violet and infra-red spectroscopy during this period, can all be followed in the pages of any national pharmacopoeia that was being published regularly during this period. This brings us to the early 1960s and at those times any regulatory interest in the quality of drugs rested almost entirely on the pharmacopoeias.

In the European Community of countries, directives have been issued to ensure that the legislation in each member state shall provide for the comprehensive examination of intended drug materials before they are allowed to enter the market. Mutagenicity, carcinogenecity, toxicity, effect of reproductive function, pharmacodynamics and pharmacokinetics are among the properties to be studied and material must be unequivocally characterized with respect to the substance itself and the amounts of impurity that are likely to arise during the course of validated production processes.

Purity has always been considered as an essential factor in ensuring drug quality. Presently drug analysis and pharmaceutical impurities are the subjects of constant review in the public interest. Pharmaceutical impurities are the unwanted chemicals that remain with the active pharmaceutical ingredients (APIs) or develop during formulation, or upon degradation of both API and formulated APIs to medicines [5]. The presence of these unwanted chemicals even in small amounts might influence the efficacy and safety of the pharmaceutical products. Therefore, for identification and quantification, the drug registration authorities have suggested the following steps:

- The quality of starting materials, reagents and solvents used during synthesis, chemical reactions involved in the synthesis, reaction conditions, purification steps and storage of the final drug substance affect the impurity profile of a drug

substance. Any minor change in the above conditions may dramatically change the impurity profile.

- It is required to detect impurities in drug substance obtained from batches manufactured during the development process, batches from the commercial process and stress conditions.
- The structures of impurities should be elucidated when present at level higher than 0.1% or in some cases higher than 0.2%, depending on daily-recommended dosage.
- The impurities are synthesized based on the suggested structures.
- The synthesized impurities are used as an impurity standard for the development of a selective analytical method for its quantitation in drug substance and / or products.

Impurities associated with APIs are classified into three groups for regulatory purposes as organic, inorganic and residual solvents [6].

- Organic impurities may arise from starting materials (most often from isomeric impurities), synthetic intermediates (incomplete reaction or excess reagent used), by-products, degradation products, reagents, ligands and catalysts. The reagents, ligands and catalysts are less commonly found in APIs; in some cases they may pose a problem as impurities.
- Inorganic impurities present in pharmaceutical products originate from the equipment used and from reagents, catalysts, heavy metals, drying agents and filter aids. The chances of having impurities from reagents, ligands and catalysts are rare: however, in some processes, these could create a problem unless the manufacturers take proper care during production. The main sources of impurity of heavy metals are the water used in the processes and the reactors (if stainless

steel reactors are used), where acidification or acid hydrolysis takes place. These impurities of heavy metals can easily be avoided using demineralized water and glass-lined reactors.

- Residual solvents and other volatile impurities must be detected and assayed, not only because of their potential toxicity and deleterious environmental effects, but also because they can impart undesirable organoleptic characteristics to drugs. Since residual solvents arise in excipients and occasionally in the manufacture of drug products. According to ICH guidelines, residual solvents can be grouped into three categories based on the possible risk to human health [6]. Category I incudes solvents such as benzene (2 ppm limit) and carbon tetrachloride (4 ppm limit). The solvents belong to Category II are methylene chloride (600 ppm limit), methanol (3000 ppm limit), pyridine (200 ppm limit), toluene (890 ppm limit), N,N-dimethyl formamide (880 ppm limit) and acetonitrile (410 ppm limit). The solvents of category II are most commonly used during the manufacturing process. Acetic acid, acetone, isopropyl alcohol, butanol, ethanol and ethyl acetate are solvents of category III. These solvents have higher tolerance limits. ICH guidelines [7] have recommended daily exposures of 50 mg or less per day.

ICH guidelines achieved a great deal in harmonizing the definitions of the impurities in new drug substances. It is necessary to perform all the investigations on appropriate reference standards of drugs and impurities to get meaningful specifications. In order to meet the challenges to ensure high degree of purity of drug substances and drug products, a scheme is proposed for profiling drug impurity (**Fig. 1.1**).

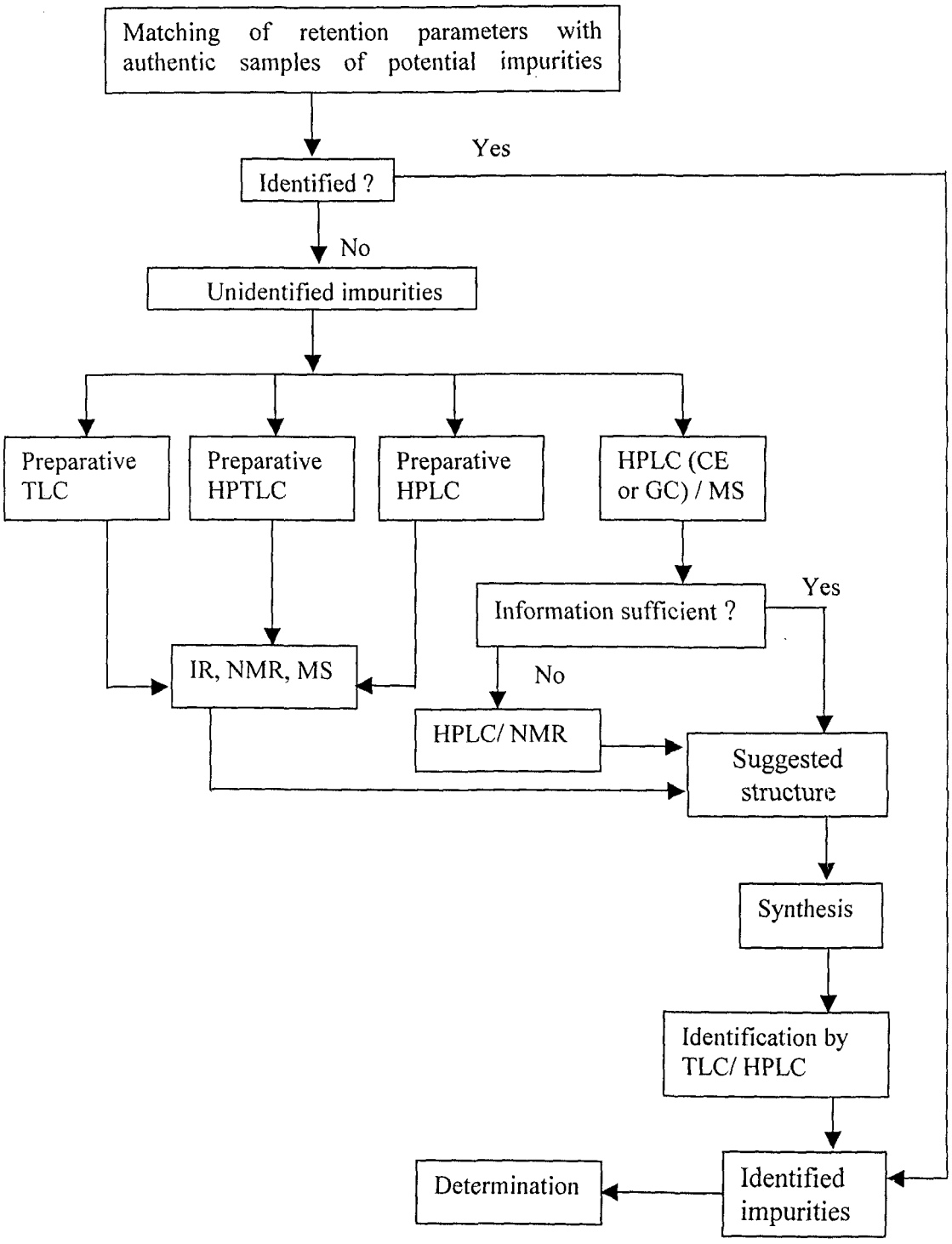


Fig. 1.1. Proposed chart for profiling drug impurity.

Over the last 30 years, the very existence of searching analytical methods has contributed quite considerably to an improvement in the general quality of the drugs. We are all familiar with adage that "Quality must be built into a product - it can never be analyzed into it". The most frequently used analytical techniques in pharmaceutical analysis are titrimetry, chromatography, electrochemical methods and spectroscopy. Sometimes these techniques are combined with other supporting/ subsidiary instruments like flow injection analysis system or kinetic type of analysis. Thus making them more sensitive, selective and fast.

In the field of drug analysis volumetric methods, either direct or indirect, have their own importance due to their inherent simplicity. This is the reason why still a number of official methods for the determination of pharmaceuticals in the pharmacopocieas are based on titrimetry. Though it is the oldest technique in the market now but still having their recognition in the field of scientific research. Recently titrimetric methods have been used for the determination of albendazole [8], gatifloxacin [9], promethazine theodate [10] and procaine hydrochloride [11] in commercial dosage forms.

Chromatographic methods have many applications in trace analysis and sometimes prove it as the only way. Chromatography is used extensively in the pharmaceutical industry as a separation tool for qualitative and quantitative analysis of various pharmaceutical compounds and drugs [12]. The different types of chromatography such as thin layer chromatography, high performance thin layer chromatography, column chromatography, high performance liquid chromatography, gas chromatography and capillary electro-chromatography have most frequently used in the field of pharmaceutical as well as biomedical analyses.

Thin layer chromatography (TLC) has enjoyed widespread popularity in modern pharmaceutical analysis because of its simplicity, requires minimal instrumentation, laboratory space and maintenance. However, to achieve good precision, accuracy, reproducibility and speed of analysis, a certain degree of instrumentation is required: the use of densitometric evaluation is necessary at least for quantification [13-17]. A TLC method has been developed for the quantitation of theophylline in plasma using plates coated with silica gel 60 F254 [18]. In an effort, resolution of three commonly used β-blockers, (±) atenolol, (±) metoprolol and (±) propranolol, into their enantiomers has been achieved using normal-phase TLC on silica gel G plates impregnated with L-aspartic acid as the chiral selector with different combinations of acetonitrile-methanol-water as mobile phase [19]. Thin layer chromatography has also been employed to identify an unknown compound in hydroquinine that has a higher R_f value and forms during migration when dichloromethane and methanol are used as the development solvent [20]. The level of quinine, cinchonine and cinconidine in natural products and marketed formulations has been determined by TLC in combination with fluorescence enhanced detection [21]. This technique has also been used to analyse polymyxin B, framycetin, and dexamethasone in an ointment [22] and the contents of drotaverine and nifuroxazide in capsules [23].

High performance thin layer chromatography (HPTLC) is an Off-Line technique whose every stage of analysis can be visualized. It is a relatively young thin-layer technique, which is still undergoing improvements and gaining the popularity. The advantages [24] of HPTLC are given below:

- simple to handle the instrument

- short analysis time to analyze complex or crude samples with minimum sample clean up
- optimized coating material with a separation power superior to that of the best HPLC separation material
- an improved method of feeding the mobile phase
- evaluate the entire chromatogram with a wide variety of techniques and parameters without time constraints
- simultaneous but independent development of multiple sample and standards on each plate, leading to an increased reliability of results (in-system calibrations)
- robustness for easy transfer of samples.

Several drugs have been successfully investigated by HPTLC in pharmaceutical preparations [25-28].

At present, HPLC is the most widely used technique for the analysis of bulk drugs and their formulations [29,30]. Derivatization of the drugs prior to analysis is normally not required. The sample preparation is extremely simple and the errors associated with it are generally kept to a minimum by using HPLC. Gradient elution, temperature and wavelength-programming techniques provide valuable information regarding the undetected components of a given drug. Generally speaking, gradient elution, although extensively used in pharmaceutical research, is not popular because many of the above advantages are lost. Instead, screening for potential impurities is often performed by a combination of isocratic HPLC methods. For example the search for 11 potential impurities of mizolastine required the use of three isocratic HPLC methods because of the large difference in the hydrophobicities of the impurities [31]. The choice of proper detection mode is crucial to ensure that all the components are detected. With UV detection, this

problem could be overcome by using a multiple wavelength-scanning program, which is capable of monitoring several wavelengths simultaneously. It provides assurance that all the UV- absorbing components are detected, if present in sufficient quality. Chiral detectors are useful in determining the purity of enantiomeric drugs by HPLC. Several books and articles have dealt the theory and practices of HPLC [32,33]. Many research papers have described the latest developments in its instrumentation and applications in the pharmaceutical industries [34,35]. However, the limitations of HPLC include the cost of columns, solvents and a lack of long-term reproducibility due to the proprietary nature of column packings.

Because of the specificity and sensitivity afforded by gas chromatography (GC), it has been widely used for the detection and determination of pharmaceuticals in dosage forms and biological fluids [36-39]. The use of mass spectrometer (MS) as the gas chromatographic detector provides additional sensitivity and specificity over other analytical techniques [40]. Gas chromatography-mass spectrometry is utilized for monitoring anticancer drugs [41] and also used for the determination of ramipril and ramiprilat in human plasma and urine [42]. Trace level contaminants in pure substances can also be determined by GC / MS [43].

Capillary electrophoresis (CE) is a relatively new analytical technique based on the separation of charged analytes through a small capillary under the influence of an electric field. It is based on a separation mechanism, which differs from all types of chromatography, which makes it either a potential alternative analytical technique capable of faster analysis and higher efficiency than HPLC, or complimentary technique to HPLC to augment the information obtained from the analysis. Thus, CE is becoming a powerful separation technique for large and small molecules, which can be organic and inorganic [44]. It has found several

applications in pharmaceutical analysis [45-51]. Different modes of CE such as capillary zone electrophoresis [52,53], micellar electrokinetic chromatography [54,55], isotachophoresis [56,57], capillary-electrokinetic [58,59], capillary gel electrophoresis [60,61], iso-electric focusing [62,63] and affinity capillary electrophoresis [64, 65] have been developed and applied for pharmaceutical purity testing and in bioanalysis of drugs.

The use of electrochemical methods for the analysis of drugs and other compounds of biological interest has increased greatly over the last few years. This is due to the availability of a wide variety of electrode materials, electrochemical cells, electrochemical instruments, and electrochemical techniques that can be used with convenience and reliability are now commercially available. Electrochemical methods are characterized by high sensitivity, selectivity and accuracy. Pharmaceuticals containing phenolic, amino, heterocyclic nitrogen, ketonic or aldehyde group undergo oxidation at their characteristic potentials and hence selectivity of the technique is increased. Important electrochemical techniques such as amperometry, conductometry, potentiometry, polarography, differential pulse voltammetry, square wave voltammetry, cyclic voltammetry, anodic and cathodic stripping voltammetry have been used in drug analysis [66]. Potentiometric titrations are also reported in the pharmacopoeia as the standard method for the determination of certain drug substances [67-69]. Despite the wide use of spectrometry and chromatography, analysts are choosing polarography and voltammetry [70-75] as an alternative due to their selectivity and sensitivity.

Spectroscopic methods are widely used for the assay of drugs in pharmaceutical formulations. The simplicity of UV-spectrophotometry is known and most widely accepted for qualitative and quantitative analysis and in structure

identification. UV- spectrophotometry, usually, does not require elaborate sample preparation steps prior to assay. Therefore, the sample can be recovered for further testing or subsequent analytical procedures. However, these methods are inadequate when two or more drugs showing similar UV spectra in the presence of other components present in the drug sample like excipients or decomposition products. The use of UV-spectrophotometry for drug analysis in pharmaceutical prepartions has been increased rapidly in the last few years [76-79].

Photometric methods of analysis are performed in the visible region of light. These methods are usually based on the following aspects:

- complex-formation reaction
- oxidation-reduction process
- a catalytic effect

In each type of reaction the absorbance of coloured compounds is measured. Usually the analyte under investigation being colourless, they are reacted with suitable chemical reagents in order to convert them in coloured compounds. Simple colourimetric and UV methods continue to be popular for carrying out single-component assays on a variety of formulated products. The examples of UV-visible spectrophotometric methods for the quantiation of pharmaceutical compounds that have been published [80-124] are given in **Table 1.1.**

In the course of this discussion, the importance of computer-aided spectrophotometric determination of multicomponent systems cannot by ignored. Pharmaceutical preparations are usually mixtures of the active principle and various excipients that absorb in the same region as the component of interest, thereby resulting in band overlap and impending the use of the technique with simple calibration methods. The inception of microcomputers and spectrophotometers that

Table 1.1
Quantitative analysis of drugs in pharmaceutical formulations by UV-visible spectrophotometric procedures

Name of drug	Reagents used	$\lambda_{max\ (nm)}$	References
Acetoaminophen	m-Cresol	640	80
Amiodarone HCl	p-Chloranilic acid	535	81
	2,3-Dichloro 5,6- dicyano 1,4- benzoquinone	575	81
Amlodipine besylate	p-Chloranilic acid	540	82
	Ninhydrin in DMF medium	595	83
	2,3-Dichloro 5,6- dicyano 1,4- benzoquinone	580	84
	Ascorbic acid	530	84
Amoxycillin & ampicillin	KIO_3	520	85
Ampicillin, amoxycillin & carbenicillin	Folin ciocalteau phenol	750,770 & 750	86
Ascorbic acid	1-Chloro-2,4-dinitrobenzene	380	87
Benidipine HCl	Methanol	238	88
Captopril	KIO_3 in HCl medium	510	89
Diclofenac sodium	Tris buffer	284, 305	90
Diltiazem HCl	Sodium metavanadate	750	91
	Bromothymol blue	415	92
	Bromophenol blue	415	92
	Bromocresol green	415	92
Famotidine	$KMnO_4$ in alkaline medium	610	93
	Ninhydrin	590	94
Flunarizine dihydrochloride	Iodine	295, 355	95
Irbesartan	Potassium iodate and iodide in aqueous medium	352	96
Lisinopril	7,7,8,8-Tetracyanoquinodimethane	743	97
	p-Chloranilic acid	525	97
	Ninhydrin	595	98
	Ascorbic acid	530	98
Labetalol HCl	Sodium nitroprusside & hydroxylamine hydrochloride	695	99
Losartan potassium	$KMnO_4$ in alkaline medium	603	100

Levodopa	Ce(IV) nitrate in H_2SO_4 medium	510	101
Methyldopa	Ce(IV) nitrate in H_2SO_4 medium	550	101
L-dopa	NaOH	300	102
Menadione	NaOH in the presence of amine	450	103
Metoprolol tartrate	$KMnO_4$ in alkaline medium	610	104
	Ninhydrin	595	105
Mometasone furoate	Methanol	248	106
Nalidixic acid	Persulfate in alkaline medium	320,390	107
Nicorandil	Brucine-sulfanilic acid in H_2SO_4 medium	410	108
	3-Methyl-2-benzothiazoline hydrazone HCl-metol	560	108
Nifedipine	$KMnO_4$ in neutral medium	530	109
	4-Methyl amino phenol and $K_2Cr_2O_7$	525	110
	Bromocresol green	415	111
	Bromophenol blue	415	111
	Bromothymol blue	415	111
	Eriochrome Black T	520	111
	KOH in dimethylsulphoxide	430	112
	Ammonium molybdate	830	112
Norfloxacin	$KMnO_4$ in alkaline medium	603	113
Carbinoxamine	Cu (II) & eosin	538	114
Pantoprazole sodium	Potassium ferricyanide and ammonium ferric sulphate	725	115
Perindopril erbumine	1-Chloro-2,4-dinitrobenzene in dimethyl sulphoxide	420	116
Ramipril	Potassium iodate and potassium iodide in aqueous medium	352	117
Silymarin	$KMnO_4$ in neutral medium	530	118
	3-Methyl-2-benzothiazoline hydrazone & potassium persulphate	430	119
Trimethoprim	Persulphate in alkaline medium	355	120
Thyroxine	Nitrous acid	420	121
Verapamil HCl	Chloramine T	425	122
	N-Bromosuccinimide	415	123
	Potassium metaperiodate	425	124
	Tropaeolin 000 No.1	400	124

allow absorbance spectra to be expeditiously recorded at many wavelengths has enable the development of analytical methods based on the mathematical resolution of multivariate signals for the rapid quantitation of mixtures of analytes in control analyses. The application of multivariate calibration methods to spectral data in the biomedical and pharmaceutical fields has acquired a routine nature [125-128].

Partial least square has become the de facto standard for multivariate calibration because of the quality of the obtained calibration models, the ease of its implementation and the availability of software [129]. It shows the advantage of using full spectra, which is critical for the spectroscopic resolution of complex mixtures of analytes. It allows for a rapid determination of components, usually with no need of a prior separation. An additional advantage of robust multivariate methods is that calibration can be performed by ignoring the concentration of all other components except the analyte of interest. This makes these methods especially appealing for the determination of the active components in ophthalmic and nasal solutions as well as in the syrups, whose excipients may show absorption spectra that are severely overlapped with those from the analytes. The complementary use of partial least square multivariate calibration and artificial neural networks for the simultaneous spectrophotometric determination of three active components such as chlorpheniramine, nephazoline and dexamethasone in a pharmaceutical formulation has been successfully explored [130].

Difference spectrophotometry is an important and useful technique used in the determination of medicinal substances by eliminating specific interference from the degradation products, co-formulated drugs and non-specific irrelevant absorption from the formulation matrix. The technique involves reproducible attraction of the spectral properties of the absorbance difference between two solutions, provided that

the absorbance of the other absorbing substance is not affected by the reagent(s) used to alter the spectral property [131].

Near infra red (NIR) spectroscopy is a technique, which has found its way into pharmaceutical control laboratories in recent years for raw material identification, water analysis, and other pharmaceutical analyses [132-137]. There are several advantages associated with this technique such as reduction in the cost of testing, require no reagents, associated reagents preparation steps, sampling preparation steps and generally require only one working analyst day to complete testing. The US pharmacopoeia has proposed guidelines [138,139] for this technique.

Nuclear magnetic resonance (NMR) spectroscopy has been mainly used for the elucidation and confirmation of structures. For the last decade, NMR methods have been introduced to quantitative analysis in order to determine the impurity profile of a drug, to characterize the composition of drug products in body fluids, in solid state measurements to provide the information about polymorphism of drug powders, quantification of drugs in tablets [140-146] and for micro-imaging to study the dissolution of tablets [147-149].

Fluorimetry and phosphorimetry find wide applications in quantitative studies of rates of degradation, metabolism, and excretion of drugs where other analytical techniques are not sufficiently sensitive. A gradual increase in the number of papers on the potential applications of fluoimetry and phosphorimetry in the field of pharmaceutical analysis has been noticed during the last few years [150-153].

The analytical measurement of elemental concentrations is important for the analysis of the major and minor constituents of pharmaceutical products. Metals are the major constituents of several pharmaceuticals such as dialysis solutions, lithium carbonate tablets, antacids and multi-vitamin and mineral tablets. The metal ions in

pharmaceuticals are determined more accurately and conveniently by atomic absorption spectrometry. The use of atomic absorption spectrometry in this regard has been the subject of several reviews and papers [154-159].

In the recent period, analysts are much interested in coupling the chromatographic techniques with spectroscopic techniques. The advent of liquid chromatography with mass spectrometry [160-162], gas chromatography with tandem mass spectrometry and liquid chromatography-electrospray tandem mass spectrometry are good examples. Hirsch and coworkers have determined the antibiotics using chromatography-electrospray tandem mass spectrometry [163]. Dielectric relaxation spectroscopy and X-ray powder diffractometry are also utilized for the identification of pharmaceuticals [164].

One of the significant analytical developments in the last two decades has been the wide spread utilization of flow injection analysis (FIA) assay of drugs in pharmaceutical laboratories. It has several advantages:

- reduced reagent consumption
- high sampling frequency
- safety in applying toxic reagents because the whole analysis proceeds in a closed system

An additional advantage observed in flow injection analysis is increased selectivity when the analyte is accompanied by more slowly reacting compound. This technique has been utilized successfully in the determination of some compounds of pharmaceutical interest [165-170].

Kinetic automatic methods are good choices for drug analyses as they permit sensitive and selective determination of many drugs within a few seconds with no pretreatment. The principles and applications of the kinetic methods have been

reviewed [171-173]. Essentially, kinetic methods rely on the measurements of concentration changes (detected via signal changes) in a reactant (which may be the analyte itself) with time after the sample and reagents have been mixed. The sample and reagent can be mixed manually or automatically. Only slow enough reaction tolerate manual mixing and even so, they are better handled automatically, not only to obtain more rapid and reproducible results, but also to increase the reaction rate in some cases. There are several approaches that can be used for the determination of single components in the absence of any kinetic interference **(Table 1.2).** However fixed-time and initial rate methods have been used more frequently for the determination of drugs in pharmaceutical formulations and biological fluids [174-177]. Kinetic automatic techniques are generally based on open systems among the most popular of which are stopped flow system [178] and the continuous addition of reagent (CAR) technique [179-181]. Several drugs have been determined by using the CAR technique with photometric [182,183] and fluorimetric detection [184]. The use of catalysts to accelerate analytical reactions is feasible with both reaction rate and equilibrium determinations. In this concern, the use of micellar media in kinetic method is recently encouraged to enhance the rate of reaction (through micellar catalysis) and may additionally improve the sensitivity and selectivity which in turn reduce the analysis time for the analyte [185-187]. Multicomponent kinetic determinations, often called as differential rate methods, are also receiving popularity in the field of pharmaceutical research [188,189]. Two new approaches i.e. kinetic wavelength pair method [190] and H-point standard addition method [191] have been proposed for dealing with overlapping spectra of components in the binary mixtures.

Table 1.2
Single-component methods without error compensation

A. Direct-computation methods	B. Curve-fitting methods
1. Integral methods	1. Integral methods
a. Fixed-time	a. Linear responses
i. One-point	b. Non-linear response
ii. Two-point	i. Direct computation
iii. Multipoint	ii. Predictive (extrapolation)
b. Variable-time	2. Rate methods
i. One-point	
ii. Two-point	
2. Rate methods	
a. Initial-rate	
b. Intermediate-rate	
3. Integrated-signal methods	

Whenever the question of mathematical and statistical treatment arises, the role of chemometrics cannot be ignored. Chemometrics is the chemical discipline concerned with the application of mathematical, statistical methods as well as those other methods based on mathematical logic to chemistry in order to design or select optimal procedures and experiments, and provide maximum chemical information by analyzing chemical data [192,193]. Several reviews have been reported on applications of chemometric methods in biomedical and pharmaceutical analysis [194-198].

PROCESS MONITORING AND VALIDATION

Before an analytical method can be used for routine analysis, it must first be demonstrated that the method fulfills certain performance criteria. When this has been documented, the method is said to be validated. In order to address the performances of the analytical procedure adequately, the analyst is responsible to identify the relevant parameters, to design the experimental validation studies and define the appropriate acceptance criteria. The purpose of method validation is to establish that an accurate precise and rugged method has been developed. The process for the development, validation and use of analytical method is shown in **Fig. 1.2.**

The ability to provide timely, accurate, and reliable data is central to the role of analytical chemists and is especially true in discovery, development and manufacture of pharmaceuticals. Analytical data are used to screen potential drug candidates, aid in the development of drug synthesis, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. The quality of analytical data is a key factor in the success of a

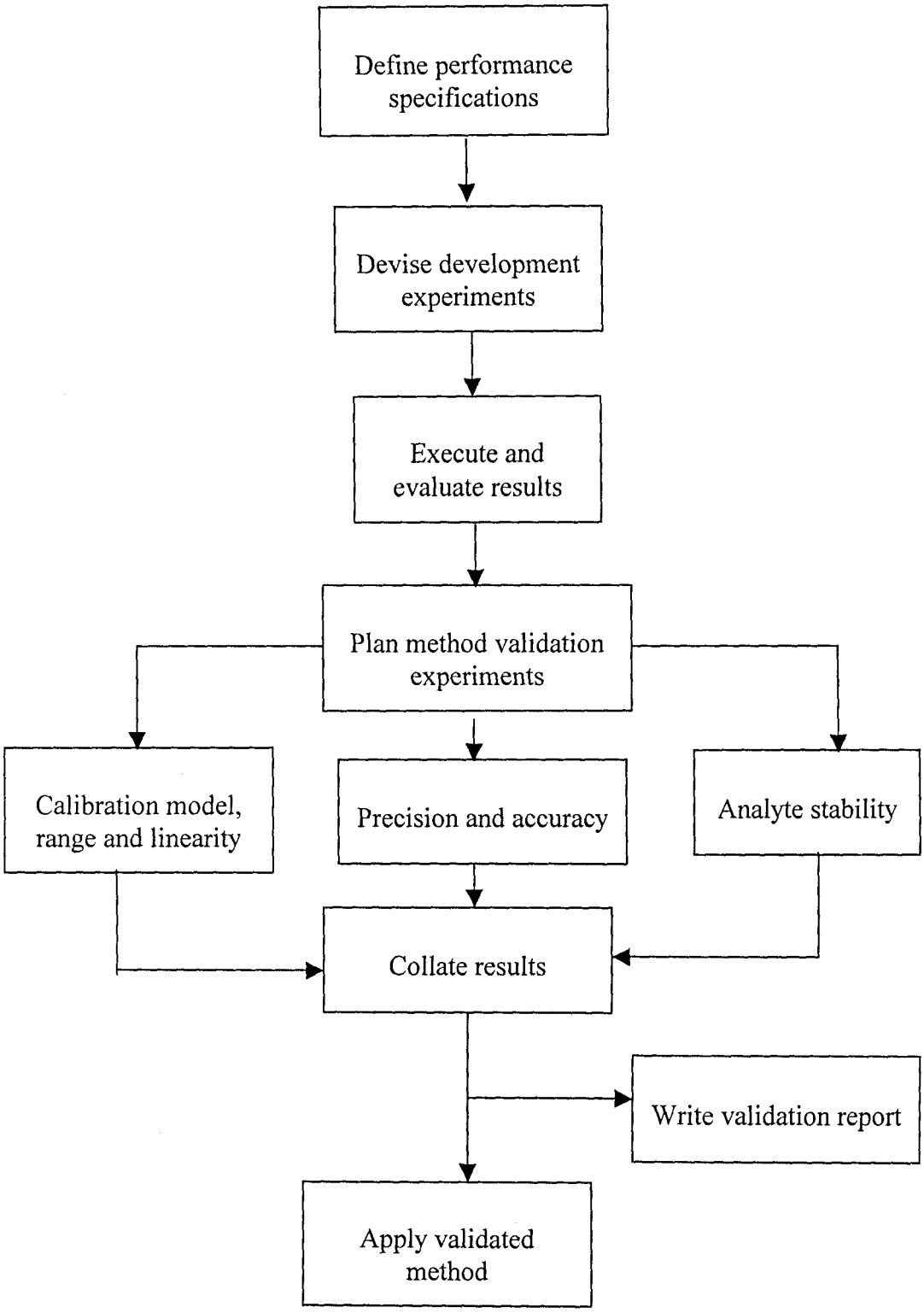


Fig. 1.2. The process of development, validation and routine use of an analytical method.

drug development programme. The process of method development and validation has a direct impact on the quality of these data.

Several International Organizations and Regulatory Authorities, which are involved in fixing the criteria for the validations, are listed in **Table 1.3**. In the field of analysis, it is very clear that the definitions cover the entire field of analytical chemistry from bioanalysis to substance and product analysis. It has been agreed that the parameters for evaluation of method reliability and overall performances are:

- confirmation of identity
- solution stability
- selectivity/ specificity
- linearity
- accuracy and precision
- limits of detection and quantitation
- recovery
- robustness/ ruggedness
- equivalence testing

The overall validation strategy consists of four components, which are prevalidation, validation proper, study proper and statistical analysis.

Among all of the International Organizations, the ICH guidelines achieved a great deal in harmonising the definitions of the required validation characteristics and their basic requirements. The International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has harmonized the requirements in two guidelines [199,200]. The first one summarizes and defines the validation characteristics needed for various types of

Table 1.3
Validation of analytical methods: International definitions

Organization	Applicability	Remarks
IUPAC	Worldwide	
ILAC	Worldwide	
WELAC	Europe	
ICH	Europe, Japan, USA	Only pharmaceutical products
ISO	Worldwide	Lack definition of selectivity and specificity

Abbreviations:
IUPAC, International Union of Pure and Applied Chemistry

ILAC, International Laboratory Accreditation Conference

WELAC, Western European Laboratory Accreditation Co-operation

ICH, International Conference on Harmonisation

ISO, International Organization for Standardization

test procedure. The second one extends the previous test to include the experimental data required and some statistical interpretation. These guidelines serve as a basis worldwide both for regulatory authorities and industries and bring the importance of a proper validation to the attention of all those involved in the process submission. In order to fulfill the validation responsibilities properly, the background of the validation parameters and their consequences must be understood. The validation characteristics and their minimum number of determinations required if applicable are given in **Table 1.4** [200].

Confirmation of identity

In general analytical methods consist of a measurement stage, which may be preceded by an isolation stage. It is necessary to establish that the signal or reaction produced at measurement stage is only due to the analyte and not due to something chemically or physically similar or arising as a coincidence. This is the confirmation of identity. Whether or not other compounds interfere with the measurement of the analyte will depend on the effectiveness of the isolation stage if it was part of the method, as well as the specificity/ selectivity of the measurement stage.

Selectivity/ specificity

There has been some controversy regarding the technical term for this validation characteristic i.e. specificity versus selectivity [201]. The terms selectivity and specificity are often used interchangeably. A detailed discussion of these terms as defined by different organizations has been made by Vessman [202].

The term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method, which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses the method

Table 1.4
Validation characteristics normally evaluated for the different types of test procedure [199] and the minimum number of determinations required (if applicable) [200]

Validation characteristics	Minimum number	Test procedure			
		Identity	Impurities		Assay[a]
			Quantitative	Limit	
Specificity[b]	-	Yes	Yes	Yes	Yes
Linearity	5 concentrations	No	Yes	No	Yes
Range	-	No	Yes	No	Yes
Accuracy	9 determinations over 3 concentration levels (e.g. 3 X 3)	No	Yes	No	Yes
Precision					
Repeatability	6 determinations at 100% or 9 determinations over 3 concentration levels (e.g. 3 X 3)	No	Yes	No	Yes
Intermediate Precision/ reproducibility[c]	2-series	No	Yes	No	Yes
Detection limit	-	No	No[d]	Yes	No
Quantitation limit	-	No	Yes	No	No

[a]Including dissolution, content potency

[b]Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure (s).

[c]Intermediate precision sufficient for submission.

[d]May be needed in some cases.

is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

In addition, where it is unclear whether or not the interferences are already present, the selectivity of the method can be investigated by studying its ability to measure, compared to other independent method/ techniques. These parameters apply to both qualitative and quantitative analysis.

Accuracy and precision

Accuracy expresses the closeness of a result to a true value. Method validation seeks to quantify the likely accuracy of the results by assessing systematic and random effects on results. Accuracy is, therefore, normally studied as two components: 'trueness' and 'precision'. The trueness (of a method) is an expression of how close the mean of a set of results (produced by the method) is to the true value. Trueness is normally expressed quantitatively in terms of "bias" which provides a measure of systematic or determinate error of an analytical method. Precision is a measure of how close results are to one another, and is usually expressed in terms of standard deviation, relative standard deviation and variance.

Generally, the precision of an analytical method is readily obtained by simply repeating the measurements. The two most common precisions are:

- repeatability (Intra day assay)
- reproducibility (Inter day assay)

They represent two extreme measure of precision, which can be obtained. Repeatability, also termed 'Intra day assay' precision (the smallest expected precision) will give an idea of the sort of variability to be expected when a method is performed by a single analyst on one piece of equipment over a short time scale by applying the whole analytical procedure to the sample i.e. the sort of variability to be

expected between the results when a sample is analysed in duplicate. If a sample is to be analysed by a number of laboratories for comparative purposes then a more meaningful precision measure is reproducibility (this is the largest measure of precision normally encountered). It is also termed as 'Inter day assay'. Both repeatability and reproducibility are generally dependent on analyte concentration, and should be determined at different concentration levels and if relevant, the relationship between precision and analyte concentration should be established.

Repeatability and intermediate precision can be calculated by an analysis of variances [203,204]. No acceptance limit for accuracy and precision for an assay method is reported in the main regulatory guidelines [205,200,206], and only a document of the European community commission deals with the performance of analytical methods and the interpratation of results [207]. Several approaches discussed in the ICH guideline are given in **Table 1.5.**

Linearity and calibration curve

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformation, proportional to the concentration of analytes in samples within a given range, is widely used in pharmaceutical analysis [208].

Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level. The requirements and relevant parameter for the various calibrations are given in **Table 1.6.**

Table 1.5
Quantitative approaches to demonstrate accuracy according to ICH [200]

Drug substance	Application of the analytical procedure to a reference material. Comparison of the results with those of a second, well characteristic procedure.
Drug Product	Application of the analytical procedure to synthetic mixtures of drug product components. Spiking of analyt to drug product. Comparison of the results with those of a second, well characterized procedure.
Impurities (quantitative)	Spiking of the impurity to drug substances or product. Comparison of the results with those of a second, well characterized procedure.

Table 1.6
Requirements for different calibration modes with relevant parameters

Quantitation	Requirements	Relevant parameters
Single-point calibration		
External standard	Linear function	Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).
	Non-significant ordinate intercept	Inclusion of zero in confidence interval of the ordinate intercept, magnitude of the intercept (as percent of the signal 100% test concentration).
	Homogenity of variances[a]	F-test of the variances at the at the lower and upper limit of the range.
Multiple-point calibration		
Linear, unweighted	Linear function	Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).
	Homogenity of variances[a]	F-test of the variances at the at the lower and upper limit of the range.
Linear, weighted	Linear function	Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic

		regression).
Non-linear	Continuous function	Appropriate equation
100 % -method (area normalization for impurities):	For main peak: linear function	Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).
	Non-significant ordinate intercept	Inclusion of zero in confidence interval of the ordinate intercept, magnitude of the intercept (as percent of the signal 100% test concentration).
	Homogenity of variances[a]	F-test of the variances at the at the lower and upper limit of the range.
	For impurities: linear function	Standard error of slope (residual standard deviation), sensitivities (relative standard deviation, graph), residual analysis, statistical tests (vs. quadratic regression).

[a] May be presumed for a limited range (factor 10-20).

Limits of detection and quantitation

The limit of detection (LOD) of a technique can be conceived of as the smallest concentration, or amount of analyte that can be established as being different, at a reasonable statistical confidence level, from a blank (a material similar is composition to the sample except that the analyte is absent) [209-211]. One common defintion of LOD is the concentration (or quantity) of analyte that produces a signal that exceeds the signal observed from a blank by an amount equal to three times the standard deviation for the measurement on the blank [211, 212]. At analyte concentrations at or near the LOD, the precision of the measured data is usually poor. Thus, it is inadvisable to attempt to quantify the analyte unless its concentration is well above the LOD.

A limit of quantitation (LOQ), defined as the analyte concentration for which the signal exceeds that for a realistic analytical blank by 10 times the standard deviation, is often specified as the smallest analyte concentration that one should attempt to quantify [211,212]. Several approaches have been given in the ICH guidelines to determine the detection and quantitation limit (**Table 1.7.**).

Range of analytical method

The range of analytical method is the concentration interval over which acceptable accuracy, linearity, and precision are obtained. In practice, the range is determined using data from the linearity and accuracy studies. **Fig. 1.3** illustrates the definition of the dynamic range of an analytical method, which extends from the lowest concentration at which quantitative measurements can be made (limit of quantitation, or LOQ) to the concentration at which the calibration curve departs from linearity (limit of linearity, or LOL). To be very useful, an analytical method

Table 1.7
Approaches for determining the limits of detection and quantitation [200] [a]

Approach	Detection limit	Quantitation limit
Visual evaluation	Minimum level detectable	Minimum level quantifiable
Signal-to-noise	3:1 or 2:1	10: 1
Standard deviation of the response (S_o)[b] and the slope (b)	$3.3 \times S_o / b$	$10.0 \times S_o / b$

[a] verification with a suitable number of samples.

[b] Standard deviation of the blank, residual standard deviation of the calibration line, or standard deviation of the intercept.

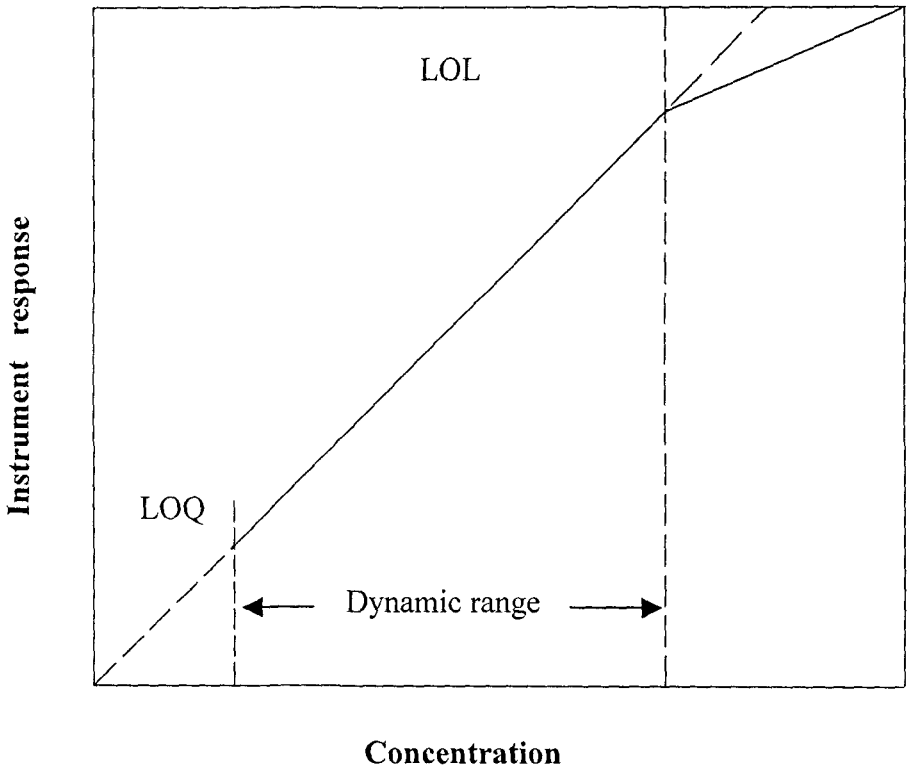


Fig. 1.3. Useful range of an analytical method: LOQ = Limit of quantitation and LOL = Limit of linear response.

should have a dynamic range of at least two orders of magnitude. Some methods have applicable concentration range of five to six orders of magnitude.

Robustness/ ruggedness

Robustness testing is now a days best known and most widely applied in the pharmaceutical world because of the strict regulations in that domain set by regulatory authorities which require extensively validated methods. International Conference on Harmonisation of Technical Requirements for Human Use (ICH) [214] defines "the robustness/ ruggedness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [215]. The term ruggedness is frequently used as a synonym [216-219]. The ICH guidelines [215] also recommend that "one consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g. resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used". Several definitions for robustness or ruggedness exist which are, however, all closely related [220-223].

The Dutch pharmacists guidelines [220], the ICH guidelines [224] as well as some authors working in bio-analysis [225] consider robustness a method validation topic performed during the development and optimization phase of a method, while others [226] consider it as belonging to the development of the analytical procedure.

Therefore, the robustness test can be viewed as a part of method validation that is performed at the end of the method development or at the beginning of the validation procedure.

Equivalence testing

An important property of an analytical method is that it should be free from the systematic error (bias). Determining bias involve analyzing one or more standard reference materials whose analyte concentration is known. However, random errors make it unlikely that the measured amount will equal the known amount even when no systematic errors are present. In order to decide whether the difference between the observed and standard values can be accounted for by random variation, a statistical test known as a significance test is used in the interpretation of analytical data.

Student's t-test

Here comparison is made between two sets of replicate measurements made by two different methods, one is the test method while other is accepted (reference method). A statistical t-value is calculated using the relation:

$$\pm t = \frac{\bar{x}_1 - \bar{x}_2}{S_p}\sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$

where

$\bar{x}_1$ = Mean from the test method

$\bar{x}_2$ = Mean from the accepted (reference) method

n_1 and n_2 = Number of measurements.

S_p = Pooled standard deviation of the individual measurements of two sets is given by

$$S_p = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

The calculated t-value is compared with a tabulated value for the given number of tests at the desired confidence level. If $t_{cal} > t_{tab}$ then there is a significant difference between the results obtained by the two methods at the given confidence level. But if, $t_{cal} < t_{tab}$ then there is no significant difference between the methods compared.

F-test

This test allows comparison of the variance i.e. the square of the standard deviation of the two methods (the new method and the accepted reference method) and indicates whether there is a significant difference between the two methods or not. If S_1 and S_2 are the standard deviations, then

$$F = \frac{S_1^2}{S_2^2}$$

where

$$S_1^2 > S_2^2$$

If $F_{cal} > F_{tab}$ at the selected confidence level, then there is a significant difference between the variances of the two methods.

Interval hypothesis

For pharmaceutical analysis, a bias of ± 2.0 % is acceptable [227] and can be calculated statistically [228]. For example, the test method (method 2) is considered acceptable if its true mean is within ± 2.0 % of that of the reference method (method 1). This can be written as

$$0.98 < \mu_2 / \mu_1 < 1.02$$

which can be generalized to

$$\theta_L \angle \mu_2 / \mu_1 \angle \theta_U$$

where θ_L and θ_U represent the lower and the upper acceptance limits, respectively, when μ_2 is expressed as a portion of the reference method μ_1. Statistically, θ_L and θ_U can be calculated from the relation:

$$\theta^2\left(\overline{x_1^2} - S_p^2 t_{tab}{}^2 / n_1\right) + \theta(-2\overline{x_1 x_2}) + \left(\overline{x_2^2} - S_p^2 t_{tab}{}^2 / n_2\right) = 0$$

The lower limit (θ_L) and the upper limit (θ_U) of the confidence interval are obtained as:

$$\theta_L = \frac{-b - \sqrt{b^2 - 4ac}}{2a}$$

$$\theta_U = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

where

$$a = \bar{x}_1^{-2} - \frac{S_p{}^2 t_{tab}{}^2}{n_1}$$

$$b = -2\bar{x}_1 \bar{x}_2$$

$$c = \bar{x}_2^{-2} - \frac{S_p{}^2 t_{tab}{}^2}{n_2}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the estimates of μ_1 and μ_2 based on n_1 and n_2 measurements respectively. S_p is an estimate of the pooled S of the measurements. t_{tab} is the tabulated one sided *t*-value, with $n_1 + n_2 - 2$ degrees of freedom at the specified level of significance.

Testing for outliers

Frequently, when a series of replicate measurements of same quantity are made, one of the results will appear too different markedly from the other. There is then a great temptation to discard this "outlier" before calculating the mean and the

standard deviation of the data or applying statistical tests to compare the data with other measurements. The best-known method used for this purpose is Dixon's Q test.

$$Q = \frac{|\text{Suspected value} - \text{Nearest value}|}{(\text{Largest value} - \text{Smallest value})}$$

If $Q_{cal} > Q_{tab}$ at a given confidence level, then the outlier can be rejected.

Statistical treatment of calibration data

The quality control sample sequence is carefully monitored for systematic errors. For each standard curve, the slope, intercept, variance, correlation coefficient and the interpolated calibrated concentrations are reported.

Acceptance of the assay results are determined by monitoring the quality control results. If the interpolated concentrations are within the control charts confidence limits, established during the method validation, the data are considered valid. Upon completing a study proper and accepting the analytical runs, the quality control results are incorporated into their respective data basis to update their confidence limits.

Correlation and regression

When using instrumental methods it is often necessary to carry out a calibration procedure by using a series of samples (standards) each having a known concentration of the analyte. Two statistical procedures should be applied to a calibration curve:

- test whether the graph is linear or in the form of a curve
- find the best straight line (or curve) through the data points

Linearity is often tested by the correlation coefficient, 'r', which can be calculated for a calibration curve to ascertain the degree of correlation between the measured instrumental variables and the sample concentration.

$$r = \frac{\Sigma x_i y_i - n\bar{x}\bar{y}}{\sqrt{(\Sigma x_i^2 - n\bar{x}^2)(\Sigma y_i^2 - n\bar{y}^2)}}$$

$$= \frac{n\Sigma x_i y_i - \Sigma x_i \Sigma y_i}{\sqrt{[n\Sigma x_i^2 - (\Sigma x_i)^2][n\Sigma y_i^2 - (\Sigma y_i)^2]}}$$

where

$\bar{x}$ = Mean of all the values of x_i

$\bar{y}$ = Mean of all the values of y_i

n = Number of data points

The maximum value of r is 1. When this occurs there is exact correlation between the two variables (x and y). When the value of r is zero (xy = 0), there is complete independence of the variables. The minimum value of r is -1, indicates that the assumed dependence is opposite to what exists. As a general rule, $0.90 < r < 0.95$ indicates a fair curve, $0.95 < r < 0.99$ as a good curve, and $r > 0.99$ includes excellent linearity.

Linear least squares

Once a linear relationship has been shown to a high probability by the value of the correlation coefficient, r, then the best straight line through the data points has to be evaluated by linear regression (the method of least square). The equation of the straight line is A= a + b C where A is usually the measured variable that is absorbance, plotted as a function of C that is the concentrations of the standards in a spectrophotometric calibration. To obtain the regression line A on C, the slope b of the line and the intercept 'a' on the Y-axis are given by following equations.

$$b = \frac{\Sigma x_i y_i - [(\Sigma x_i \Sigma y_i)/n]}{\Sigma x_i^{\,2} - [(\Sigma x_i)^2/n]}$$

$$a = \bar{y} - b\bar{x}$$

Errors in the slope and the intercept

$$S_o = \sqrt{\Sigma(y_i - \bar{y})^2 / (n-2)}$$

where $\bar{y}$ values are obtained from calculated regression line for given values of x; once the value S_o has been obtained, both the standard deviations of the slope S_b and the intercept S_a can be obtained from the following equations

$$S_b = S_o \sqrt{\Sigma(x_i - \bar{x})^2}$$

$$S_a = S_o \sqrt{\Sigma x_i^2 / n\Sigma(x_i - \bar{x})^2}$$

Error in the estimation of concentration

$$S_c = \frac{S_o}{b}\left[1 + \frac{1}{n} + \frac{(y - \bar{y})^2}{b^2\Sigma(x - \bar{x})^2}\right]^{1/2}$$

where $\bar{x}$ and $\bar{y}$ are the average concentration and absorbance values, respectively for n standard solutions.

Confidence limit

The confidence limit defined by the experiment means '$\bar{x}$' of 'n' replicate measurements and the standard deviation (s) within the range where the true value falls is given by

$$\text{C.L. for } \mu = \bar{x} \pm \frac{ts}{\sqrt{n}}$$

where

μ = true mean

t = statistical factor that depends on the number of degrees of freedom and the confidence level desired.

CLASSIFICATION OF DRUGS

All drugs may be discovered from a variety of natural sources or created synthetically in the laboratory. Therefore, drugs according to their chemical nature can be divided into organic and inorganic compounds. Medicinally important drugs can be broadly divided into two classes.

Chemical classification

The drugs are classified according to their chemical structure and properties without taking the pharmacological action under consideration. In this class most of the drugs are having at least an organic substrate, the further classification is done in the relevant manner.

Pharmacological classification

In this class the drugs are divided according to their action on the organism's organ (viz. gastrointestinal, heart, brain, lymphatic system, respiratory system, endocrine system, central nervous system etc). Hence these drugs are named like antiulcer, antianginal, narcotics, analgesics, antibiotics, diuretics, anesthetics etc. Further classification of each group is done according to the therapeutic/ pharmacological specificity with the relevant organ. A classification of the gastro-intestinal drugs with respect to their therapeutic importance for the treatment of gastric ulcers has been given in **Fig. 1.4.**

ANTIULCER DRUGS

Mankind has lived with peptic ulcers since ancient times. The term acid-peptic disorders encompass a variety of relatively specific medical conditions which injury by gastric acid (and activated pepsin) is thought to play an important role. These disorders include gastroesophageal reflux disease, benign peptic ulcers of the stomach and duodenum, ulcers secondary to the use of steroidal anti-inflammatory

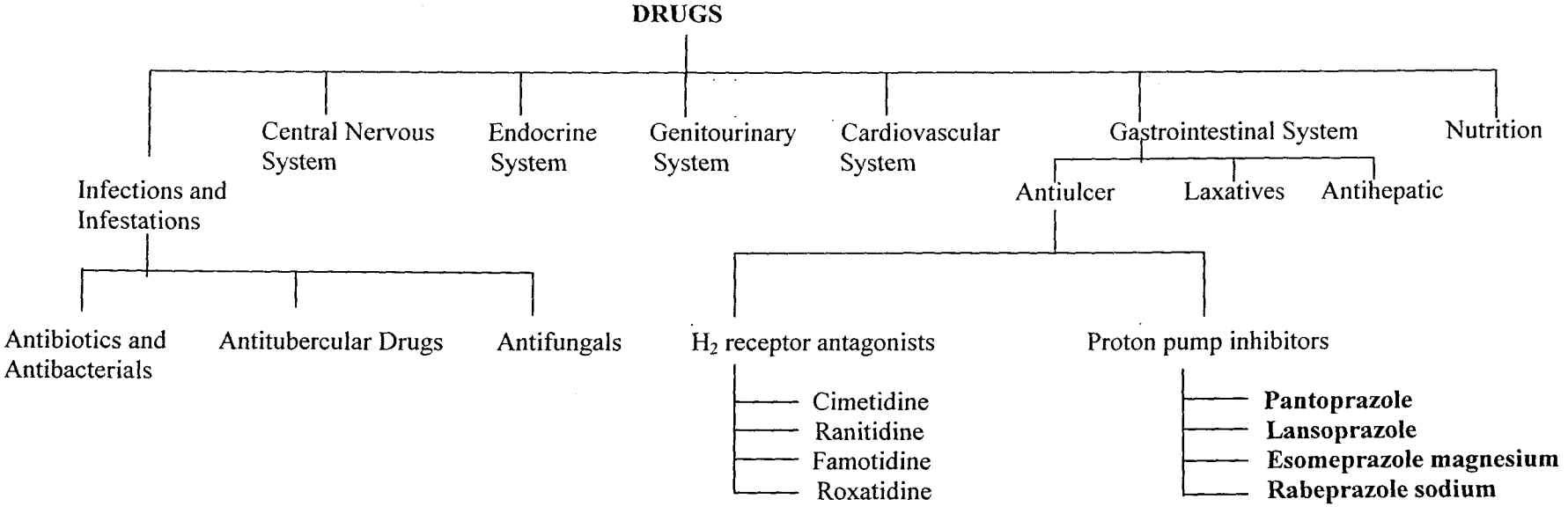
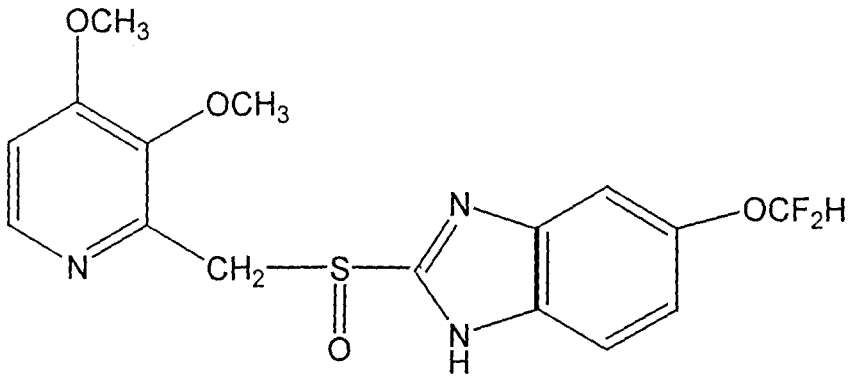


Fig. 1.4. Classification of drugs based on pharmacological action on human organs.

drugs and ulcers due to the rare Zollinger-Ellison syndrome. It appears that exposure of the involved tissue to acid is essential to the development of clinical symptoms in most instances of these diseases. Control of gastric acidity is, therefore, a cornerstone of therapy in these disorders, even though these approaches may not address the fundamental pathophysiological process. This thesis deals with quantitative analysis of certain antiulcer drugs with special reference to proton pump inhibitors (pantoprazole, lansoprazole, esomeprazole magnesium, rabeprazole sodium) in pharmaceutical formulations.

PANTOPRAZOLE ($C_{16}H_{15}F_2N_3O_4S$)

Pantoprazole, 5-difluoromethoxybenzimidazole-2-yl 3,4-dimethoxy-2-pyridylmethyl sulphoxide (CAS: 102625-70-7; MW: 383.4) is an irreversible proton pump (H^+/ K^+- ATPase) inhibitor (PPI) that decreases acid secretion from gastric parietal cells and its major metabolite is pantoprazole sulphone. It is also effective in Zollinger-Ellison syndrome and in preventing ulcer rebleeding. Thus pantoprazole is a valuable alternate to other PPIs in the treatment of acid related disorders.

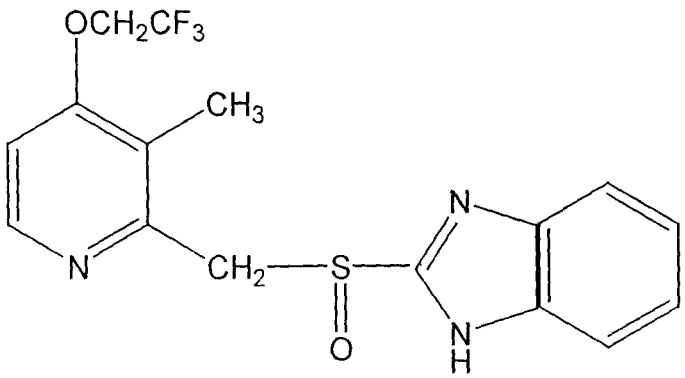


Pantoprazole

LANSOPRAZOLE ($C_{16}H_{14}F_3N_3O_2S$)

Lansoprazole is an important proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the gastric H^+, K^+ ATPase enzyme system at

the secretory surface of the gastric parietal cells. It is chemically known as 2-[[[3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridinyl]methyl]sulfinyl], 1H-Benzimidazole [CAS: 103577-45-3; MW: 369.36]. The drug is effectively useful in the treatment of duodenal ulcer, gastric ulcer, reflux oesophagitis and helicobactor pylori infection. In addition to its efficacy in healing or maintenance treatment it may provide more effective system relief than other comparator agents.

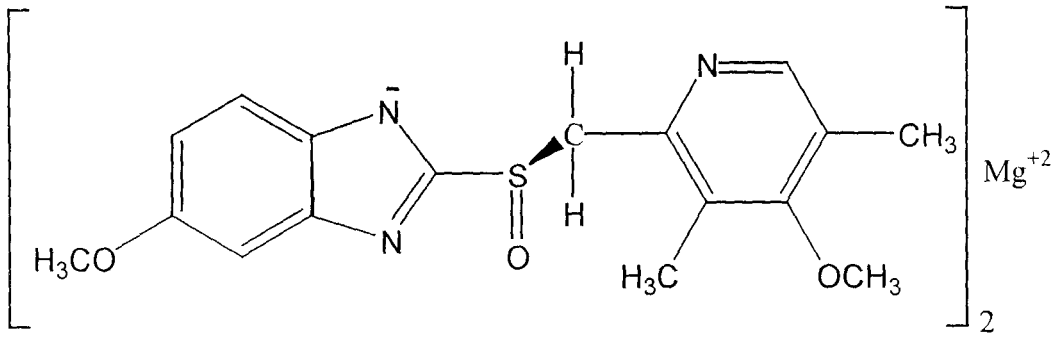


Lansoprazole

ESOMEPRAZOLE MAGNESIUM ($C_{34}H_{36}MgN_6O_6.3H_2O$)

Esomeprazole magnesium is chemically known as 5-methoxy-2-{(S)-[(4-methoxy-3,5-dimethyl-2-pyridyl) methyl] sulfinyl} benzimidazole magnesium trihydrate (CAS: 217087-09-7; MW: 767.2). This is the first proton pump inhibitor developed as a single optical isomer for the treatment of acid-related diseases. It has the advantages over omeprazole in terms of pharmacokinetic characteristics and acid suppression. The drug is used in the management of patients with gastroesophageal reflux disease, erosive reflux esophagitis and peptic ulcer. The drug is a weak base that is concentrated in the acidic compartment of secretory canaliculus of the parietal cell where it undergoes acid-catalysed transformation to a tetracyclic achiral cationic

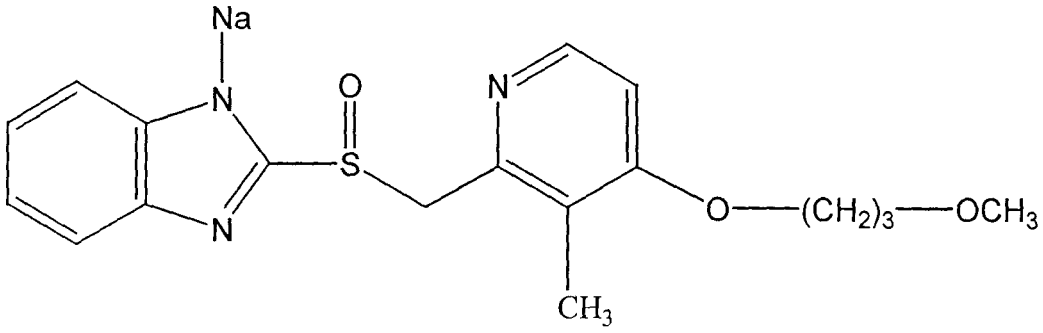
sulphenamide. This then reacts with specific cysteines resulting in the inhibitation of the H^+ / K^+- ATPase enzyme.



Esomeprazole magnesium

RABEPRAZOLE SODIUM ($C_{18}H_{20}N_3NaO_3S$)

Rabeprazole sodium is chemically known as 2-({[4-(3-methoxy propoxy)-3-methyl-2-pyridyl] methyl} sulfinyl)-1H-benzimidazole sodium (CAS: 117976-89-3; MW: 381.4). It is well-tolerated proton pump inhibitor. It has proven efficacy in healing, symptom relief and prevention of relapse peptic ulcers and gastro-oesophageal reflux disease. It is an important alternative to H_2 antagonists and an additional treatment option to other proton pump inhibitors in the management of acid related disorders. It inhibits the gastric parietal cell proton pump (H^+ / K^+-ATPase), dose dependently reducing basal and peptone stimulated acid secretion with 20 mg per day providing the optimum antisecretory effect.



Rabeprazole sodium

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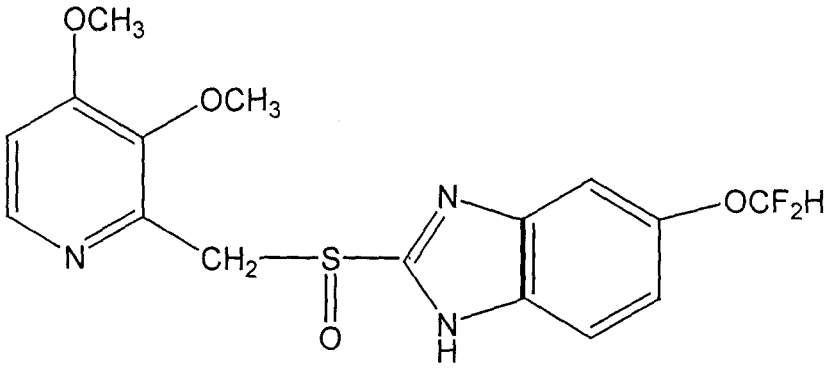
CHAPTER-2

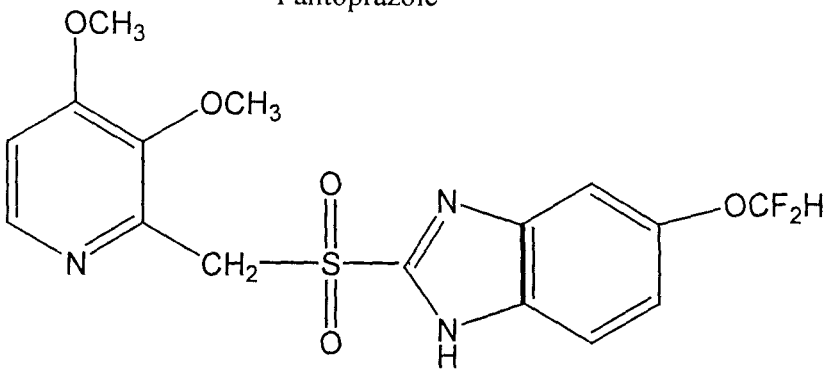
KINETIC SPECTROPHOTOMETRIC ANALYSIS OF PANTOPRAZOLE IN COMMERCIAL DOSAGE FORMS

INTRODUCTION

Pantoprazole, 5-difluoromethoxybenzimidazole-2-yl 3,4-dimethoxy-2-pyridylmethyl sulphoxide (CAS: 102625-70-7; MW: 383.4) is an irreversible proton pump (H^+/ K^+- ATPase) inhibitor (PPI) that decreases acid secretion from gastric parietal cells; its major metabolite is pantoprazole sulphone [1]. The structural formulae are shown in **Scheme 2.1**. It is also effective in Zollinger-Ellison syndrome and in preventing ulcer rebleeding. Thus pantoprazole is a valuable alternate to other PPIs in the treatment of acid-related disorders. The drug is officially listed in Martindale The Extra Pharmacopoeia [2]. The assay of drug in bulk and dosage forms is not announced in any pharmacopoeia and therefore requires much more investigation in order to assure the exact quantity of drug in pharmaceutical formulations. A literature survey reveals that high performance liquid chromatography [3-5], capillary zone electrophoresis [6], and voltammetry [7] have been employed for its quantification. The above-mentioned techniques, of course, are sensitive enough but are expensive. Spectrophotometry is the technique of choice even today due to its inherent simplicity. It is frequently used in the laboratories of the developing countries to overcome a variety of analytical problems. In the literature only a few spectrophotometric methods have been reported. Two derivative spectrophometric procedures [8,9] have been described for the determination of pantoprazole in drug formulations. The drug content in pharmaceutical preparations has been determined spectrophotometrically [10] in the visible region based on the reaction of drug with Fe(III) to form an orange-coloured chelate, which absorbed maximally at 455 nm. The drug forms a ternary complex with eosin and copper, which



Pantoprazole



Pantoprazole sulphone

Scheme 2.1

is a basis for its analysis at 549 nm [11]. The charge transfer complexation reaction between the drug and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone or iodine has been used to quantify the drug spectrophotometrically [11]. A kinetic spectrophotometric method based on the reaction of pantoprazole with 1-fluoro 2,4-dinitrobenzene in dimethyl sulphoxide medium [12] has been reported; this showed a linear response over the concentration range of 10.0 - 20.0 μg ml^{-1}.

In this chapter, a kinetic spectrophotometric method for the determination of pantoprazole in drug formulations is described. The method is based on the oxidation of pantoprazole with Fe(III) in sulphuric acid medium; Fe(III) subsequently reduced to Fe(II), which reacts with potassium ferricyanide to form a Prussian blue product, absorbing maximally at 725 nm. The initial-rate method is applied for the determination of pantoprazole. The method is optimized and validated as per the guidelines of the International Conference on Harmonisation [13].

EXPERIMENTAL

Apparatus

A Shimadzu uv-visible spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan) was used for all absorbance measurements with matched quartz cells.

Reagents and standards

All chemicals and reagents were of analytical or pharmaceutical grade. Pantoprazole was kindly supplied by Concept Pharma. Ltd. (Mumbai, India) and was used as the reference standard. A standard solution of pantoprazole (0.1%) was prepared

by dissolving 50 mg in 50 ml distilled water. Pharmaceutical formulations of pantoprazole such as Pantec-20 (Concept, Mumbai, India) and Pantodac-20 (Zydus Cadila, Ahmedabad, India) were purchased from local markets.

Ammonium ferric sulphate (3.0×10^{-3} M; Fluka Chemie AG, Switzerland) solution was prepared by dissolving 144.66 mg of ammonium ferric sulphate in 0.003 M sulphuric acid. Potassium ferricyanide (2.5×10^{-3} M; Fluka Chemie AG, Switzerland) solution was prepared in distilled water.

Recommended procedure

Aliquots of 0.05 - 0.9 ml standard solutions of pantoprazole were pipetted into a series of 10.0 ml standard volumetric flasks. To each flask, 1.7 ml of 3.0×10^{-3} M ammonium ferric suphate was added, followed by 1.5 ml of 2.5×10^{-3} M potassium ferricyanide. Then the mixture was diluted to volume with distilled water at 35±1°C. The contents of each flask were mixed well and then immediately transferred to the spectrophotometric cell. The increase in absorbance was recorded at 725 nm as a function of time for 8.0 min. The initial rate of the reaction (ν) at different concentrations was obtained from the slope of the initial tangent to the absorbance-time curve. The calibration graph was constructed by plotting the initial rate of reaction, ν *versus* concentration of pantoprazole (C, μg ml^{-1}). The amount of the drug was evaluated either from the calibration graph or from the regression equation.

Procedure for the determination of pantoprazole in pharmaceutical formulations

The quality control sample solution containing pantoprazole at a concentration of 1.0 mg ml^{-1} was prepared. The contents of 5 tablets of 20 mg strength were obtained by gentle peeling of the hard coated shells. The contents of the tablets were put in distilled

water and left for 10.0 min for complete dispersion of the drug. Then the solution was filtered through a piece of Whatmann No. 42 filter paper (Whatmann International Limited, Kent, UK) in a 100 ml standard volumetric flask. The residue was washed well with distilled water for complete recovery of the drug and then the mixture was diluted up to the mark with distilled water. The assay was completed following the proposed procedure for the determination of pantoprazole.

Procedure for the determination of pantoprazole in synthetic mixtures

Synthetic mixtures of pantoprazole were prepared by taking various excipients commonly used in tablet dosage forms with 800 µg of pantoprazole in 10 ml standard volumetric flask and tested to study the interferences of excipients such as sodium stearyl fumarate, magnesium stearate, corn starch, lactose and talc.

***Procedure for reference method* [11]**

Aliquots of 0.1 - 0.6 ml of 0.1% pantoprazole were pipetted into a series of 10 ml standard volumetric flasks. To each flask, 6.0 ml of 0.4% 2,3-dichloro-5,6-dicyano-1,4-benzoquinone was added and the mixture was diluted to volume with acetonitrile. The absorbance was measured against the reagent blank at 457 nm. The amount of the drug in a given sample was computed from the calibration graph.

Validation

The proposed kinetic method has been validated for specificity, linearity, limit of detection, precision, accuracy and recovery.

Specificity

The reference standard and quality control samples of pantoprazole were subjected to stress conditions of light, heat, acid, base and oxidants. Each stressed

sample was measured to determine the content of pantoprazole and the results were compared to those for an unstressed time zero reference solution. The reference assay value for each unstressed product was evaluated and the contents of degradation in the stressed and control samples were estimated relative to this assay value.

Linearity

The linearity was evaluated with 10 standard solutions: 5.0, 10.0, 15.0, 20.0, 35.0, 50.0, 60.0, 70.0, 80.0 and 90.0 μg ml^{-1}. The determination was repeated five times at each concentration level.

Limits of detection (LOD) and quantitation (LOQ)

The limits of detection (LOD) and quantitation (LOQ) for the assay were calculated using the equations [14]:

$$LOD = 3.3 \times S_0 / b \text{ and } LOQ = 10 \times S_0 / b$$

where S_0 and b are the standard deviation and the slope of the calibration line.

Accuracy and precision

Intra-day precision and accuracy of the proposed method were evaluated by replicate analysis (n = 5) of calibration standards at three concentration levels (20.0, 50.0, and 80.0 μg ml^{-1}). Inter-day precision and accuracy were determined by assaying the calibration standards at the same concentration levels on five consecutive days. Precision and accuracy were based on the calculated relative standard deviation (RSD, %) and relative error (RE, %) of the found concentration compared to the theoretical one, respectively.

Recovery studies

The recovery of pantoprazole from commercial dosage forms was estimated by the standard addition method. For this purpose, a volume of 2.0 ml (or 5.0 ml) of sample solution was spiked with 2.0, 4.0, 6.0 and 7.0 ml (or 1.0, 2.0, 3.0 and 4.0) of reference standard solution (1.0 mg ml^{-1}) in a 100 ml standard volumetric flask and the mixture was diluted up to the mark with distilled water. Each level was repeated 5 times. The nominal value was determined by the proposed procedure.

Evaluation of bias

The point and interval hypothesis tests have been performed to compare the results of the proposed method with those of the reference method at 95% confidence level. The bias was evaluated by an interval hypothesis test based on the mean values of the proposed method (method 1) and the reference method (method 2). The test method is considered acceptable when its true mean is within ± 2.0 % of that of the reference method. This can be written as

$$0.98 < \mu_2 / \mu_1 < 1.02$$

which can be generalized to

$$\theta_L < \mu_2 / \mu_1 < \theta_U$$

where θ_L and θ_U are lower and upper acceptance limits, respectively which were calculated from the following quadratic equation [15].

$$\theta^2\left(\overline{x_1^2} - S_p^2 t^2 / n_1\right) - \theta\overline{x_1 x_2} + \left(\overline{x_2^2} - S_p^2 t^2 / n_2\right) = 0$$

RESULTS AND DISCUSSION

Dialkyl/diaryl/alkyl-aryl sulphoxides undergo oxidation with a number of oxidants [16,17] to form sulphone derivatives. Pantoprazole is a sulphoxide derivative that is oxidized in a similar manner by ammonium ferric sulphate in moderately acidic medium to form pantoprazole sulphone [3], and itself reduces to Fe(II). The reduced Fe(II) immediately reacts with potassium ferricyanide, resulting in the formation of Prussian blue product [18], which absorbs maximally at 725 nm. The absorbance of the coloured solution increases with time and hence, a kinetically-based spectrophotometric method was elaborated to assay the pantoprazole in pharmaceutical formulations. The various experimental parameters affecting the formation of coloured product were optimized and used throughout the experiment.

TLC study of pantoprazole and its oxidized product

To identify pantoprazole and pantoprazole sulphone, we used thin layer chromatography. The reference standard solutions of pantoprazole or of pantoprazole sulphone was applied on TLC plates coated with silica gel G (Merck, India) and developed in chloroform-methanol (10:0.7 v/v) solvent system. The plates were freed from mobile phase, dried and spots were detected in iodine chamber. The R_f values were 0.54 and 0.66 for pantoprazole and pantoprazole sulphone, respectively. The R_f value (0.66) of the reaction product confirmed the presence of pantoprazole sulphone.

Optimization of Variables

The optimum conditions for the proposed method responsible for the formation of the blue product were studied and maintained throughout the experiment.

Effect of temperature

The effect of temperature on the initial rate of reaction was studied at 303, 308, 313 and 318 K. The absorbance-time curves showed that the reaction rate increases with increase in temperature. At temperature > 313 K, the linear dynamic range of determination decreases. The linear dynamic range, regression equation and correlation coefficient obtained at different temperatures are summarized in **Table 2.1**. The best linearity was obtained at 308 K and hence this temperature was selected as an optimum temperature for the determination process.

Effect of ammonium ferric sulphate concentration

The effect of ammonium ferric sulphate concentration on the initial rate of reaction (v) was studied in the range of 2.40×10^{-4} - 6.60×10^{-4} M keeping constant [potassium ferricyanide] = 3.75×10^{-4} M and [pantoprazole] = 2.09×10^{-4} M. The initial rate of reaction increased with increase in the concentration of ammonium ferric sulphate and became constant at 3.90×10^{-4} M, and remained as such up to 6.60×10^{-4} M. The results are summarized in **Table 2.2**. Therefore, a concentration of 5.10×10^{-4} M ammonium ferric sulphate was recommended for the determination procedure.

Effect of potassium ferricyanide concentration

The effect of potassium ferricyanide concentration on the initial rate of reaction (v) was investigated in the range of 2.50×10^{-5} -5.00×10^{-4} M. The maximum value of the initial rate of reaction was obtained with 2.25×10^{-4} M potassium ferricyanide, after which further increase in the concentration of potassium ferricyanide up to 5.00×10^{-4} M resulted in no change in the initial rate of reaction (**Table 2.2**). Thus, the concentration of 3.75×10^{-4} M potassium ferricyanide was found to be most suitable

Table 2.1
Linear dynamic range, regression equation and correlation coefficient at different temperatures

Temperature (K)	Beer's law range ($\mu g\ ml^{-1}$)	Regression equation	Correlation coefficient (r)
303	10.0 - 90.0 $n = 9$	$v = -4.615 \times 10^{-6} + 1.548 \times 10^{-5} C$	0.9993
308	5.0 - 90.0 $n = 10$	$v = 3.467 \times 10^{-6} + 4.356 \times 10^{-5} C$	0.9999
313	5.0 - 90.0 $n = 10$	$v = 2.333 \times 10^{-5} + 5.606 \times 10^{-5} C$	0.9995
318	5.0 - 70.0 $n = 8$	$v = 2.455 \times 10^{-5} + 6.559 \times 10^{-5} C$	0.9998

Table 2.2
Effect of the concentrations of ammonium ferric sulphate and potassium ferricyanide on the initial rate of reaction at [Pantoprazole] = 2.086×10^{-4}M

Ammonium ferric sulphate[a]		Potassium ferricyanide[b]	
Concentration, C (mol l^{-1})	Initial rate of reaction, v (mol l^{-1} s^{-1})	Concentration, C (mol l^{-1})	Initial rate of reaction, v (mol l^{-1} s^{-1})
2.40×10^{-4}	2.98×10^{-4}	2.50×10^{-5}	1.07×10^{-4}
3.00×10^{-4}	5.95×10^{-4}	5.00×10^{-5}	2.52×10^{-4}
3.60×10^{-4}	8.77×10^{-4}	1.00×10^{-4}	4.27×10^{-4}
3.90×10^{-4}	1.24×10^{-3}	1.25×10^{-4}	6.66×10^{-4}
4.50×10^{-4}	1.24×10^{-3}	1.75×10^{-4}	1.15×10^{-3}
5.10×10^{-4}	1.24×10^{-3}	2.25×10^{-4}	1.24×10^{-3}
6.60×10^{-4}	1.24×10^{-3}	2.75×10^{-4}	1.24×10^{-3}
		3.25×10^{-4}	1.24×10^{-3}
		3.75×10^{-4}	1.24×10^{-3}
		5.00×10^{-4}	1.24×10^{-3}

[a] Keeping constant [Potassium ferricyanide] = 3.75×10^{-4} M.
[b] Keeping constant [Ammonium ferric sulphate] = 5.10×10^{-4} M.

concentration for the determination process.

Analytical data and method validation

The oxidation of pantoprazole takes place with Fe(III) in acidic medium, resulting in the formation of pantoprazole sulphone; subsequently, reduced Fe(II) reacts with potassium ferricyanide to form the blue product. The course of the reaction was followed spectrophotometrically at 725 nm. The initial rates of reaction for different concentrations of pantoprazole at 308 K were determined from the slopes of the initial tangent to the absorbance-time curves (**Fig. 2.1**); these rates are summarized in **Table 2.3**. The kinetic equation for the reaction of pantoprazole with Fe(III) and potassium ferricyanide is written as:

$$v = \frac{dx}{dt} = k_1 C^n_{Drug} C^m_{Fe(III)} C^l_{ferricyanide}$$

Under the optimized experimental conditions, *i.e.* $C_{Fe(III)} \geq 5.10 \times 10^{-4}$ M and $C_{ferricyanide} \geq 3.75 \times 10^{-4}$ M, the reaction became pseudo-zero order with respect to the reagent concentrations.

Therefore, the above equation reduced to $v = k_1 C^n_{Drug}$ where k_1 is the first rate constant and n is the order of the reaction. The order with respect to pantoprazole was evaluated from the plot of log *v* versus log *C* and was found to be 1.

Hence, the reaction would obey the pseudo-first order condition and thus the above equation reduced to $v = k_1 C_{Drug}$.

A calibration graph was constructed by plotting the initial rate of reaction (υ) *versus* the pantoprazole concentration (*C*); the graph showed a linear relationship over the concentration range of 5.0 - 90.0 μg ml^{-1} at 308 K. The regression of initial rate

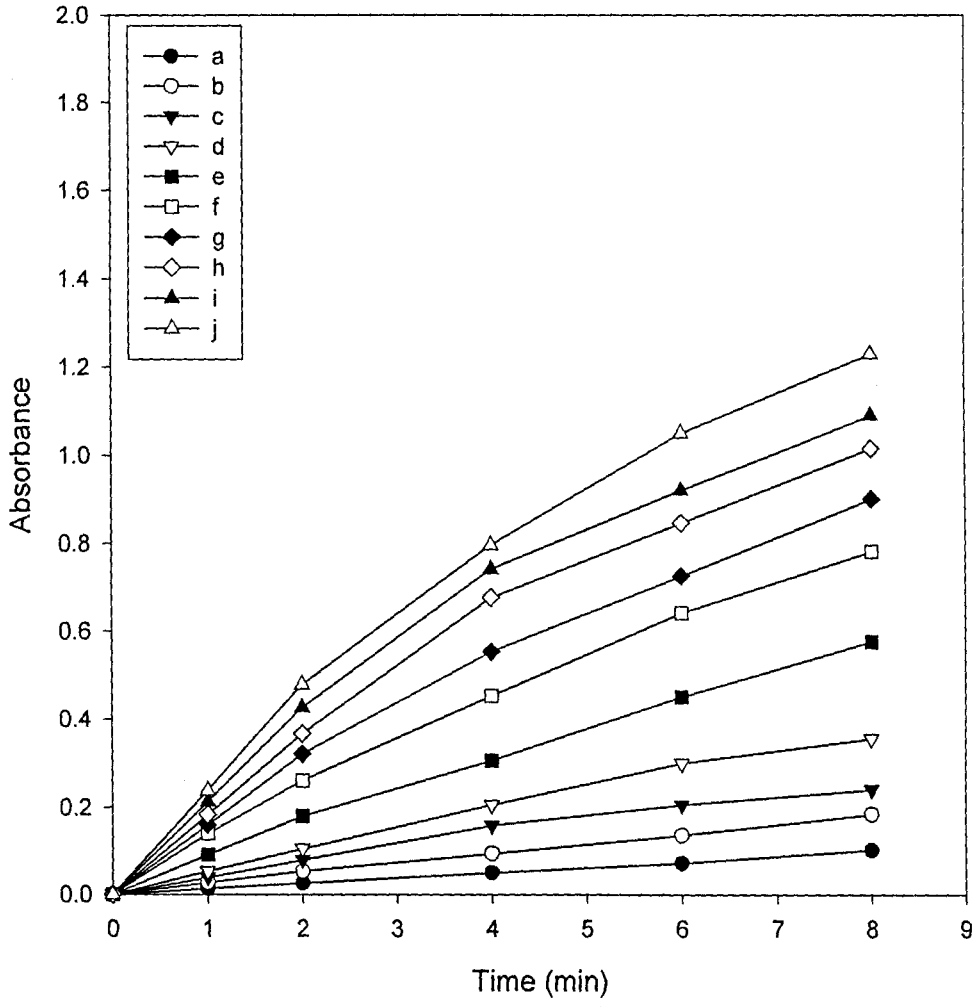


Fig. 2.1. Absorbance-time curves for the initial rate of reaction of pantoprazole: 5.10×10^{-4} M ammonium ferric sulphate and 3.75×10^{-4} M potassium ferricyanide with (a) 5.0, (b) 10.0, (c) 15.0, (d) 20.0, (e) 35.0, (f) 50.0, (g) 60.0, (h) 70.0, (i) 80.0 and (j) 90.0 μg ml^{-1} of pantoprazole.

Table 2.3
Initial rate of reaction for different concentrations of pantoprazole with [Fe(III)] = 5.10 × 10^{-4} M and [Potassium ferricyanide] = 3.75 × 10^{-4} M at 308 K

[Pantoprazole]		Initial rate of reaction, v
($\mu g\ ml^{-1}$)	($mol\ l^{-1}$)	($mol\ l^{-1}\ s^{-1}$)
5.0	1.304×10^{-5}	2.166×10^{-4}
10.0	2.608×10^{-5}	4.416×10^{-4}
15.0	3.912×10^{-5}	6.583×10^{-4}
20.0	5.216×10^{-5}	8.750×10^{-4}
35.0	9.128×10^{-5}	1.520×10^{-3}
50.0	1.304×10^{-4}	2.170×10^{-3}
60.0	1.564×10^{-4}	2.660×10^{-3}
70.0	1.825×10^{-4}	3.050×10^{-3}
80.0	2.087×10^{-4}	3.500×10^{-3}
90.0	2.347×10^{-4}	3.900×10^{-3}

versus C gave a linear regression equation, $v = 3.467 \times 10^{-6} + 4.356 \times 10^{-5} C$ with coefficient of correlation, $r = 0.9999$. The confidence limits for the slope of the line of regression and intercept were computed using the relation $b \pm tS_b$ and $a \pm tS_a$ at 95% confidence level and were found to be $4.356 \times 10^{-5} \pm 4.806 \times 10^{-7}$ and $3.467 \times 10^{-6} \pm 2.519 \times 10^{-5}$, respectively. The values of confidence limits for slope and intercept indicated the high reproducibility of the initial rate method. The limits of detection (LOD) and quantitation (LOQ) were found to be 1.46 and 4.43 μg ml^{-1}, respectively. The small value of variance (3.716×10^{-10} μg ml^{-1}) also confirmed the negligible scattering of the calibration data points around the line of regression.

Solution stability and selectivity

Pantoprazole is stable under neutral to moderately acidic conditions (pH ~ 3.5 - 7.4) [19]. The solution stability of pantoprazole was checked by observing UV spectra of pantoprazole for 14 h. The aqueous solution of the drug having λ_{max} at 298 nm showed no changes in the absorption spectra of standard and quality control sample solutions of drug for at least 14 h, when the solutions were stored at a temperature < 45°C. To identify pantoprazole and pantoprazole sulphone, we used thin layer chromatography. The standard solution, quality control sample solution and oxidized product of pantoprazole were applied on TLC plates coated with silica gel and developed in chloroform-methanol (10:0.7 v/v) solvent system. The plates were air-dried and spots were detected in the iodine chamber. In the case of standard and quality control sample solutions, a single spot was observed with $R_f = 0.54$ corresponding to pantoprazole, whereas the oxidized product showed one spot with R_f value of 0.66, confirming the presence of pantoprazole sulphone. The proposed kinetic method is a

selective one, since the major metabolite of pantoprazole *i.e.* pantoprazole sulphone does not interfere in the determination process.

Robustness

The robustness of the proposed method was investigated by challenging each operational parameter such as:

- 1.7 ml of 0.003 M ammonium ferric sulphate in 0.003 M H_2SO_4 (± 0.4 ml)
- 1.5 ml of 0.0025 M potassium ferricyanide (± 0.5 ml)
- 35°C (308 K) as the working temperature (±1°C)

Under these conditions quality control sample solutions from two commercial dosage forms claiminig 60.0 μg ml^{-1} of active pantoprazole were assayed by performing 5 independent analyses following the proposed kinetic method. The recovery results are appreciable with low values of standard deviation and relative standard deviations.

Accuracy and precision

The accuracy and precision of the proposed kinetic method were established by performing 5 independent analyses of pantoprazole in pure form at three different concentration levels (20, 50 and 80 μg ml^{-1}) by intra-day and inter-day precisions. The recovery results with relative standard deviation, standard analytical error and confidence limit are summarized in **Table 2.4**; they confirm that the accuracy and precision were acceptable. Thus the proposed method is effective for the estimation of pantoprazole.

The accuracy of the proposed kinetic method was also tested by performing recovery experiments through the standard addition method. The recovery was evaluated either by dividing the intercept by the slope value of the line of linear

Table 2.4
Test of precision (intra and inter day assays) for analyses of pantoprazole

Proposed method	Concentration ($\mu g\ ml^{-1}$)		RSD[a] (%)	SAE[b]	C.L.[c]
	Taken	Found ± SD[a]			
Intra day assay	20.0	20.01 ± 0.05	0.24	0.021	0.059
	50.0	49.95 ± 0.09	0.18	0.041	0.113
	80.0	80.00 ± 0.08	0.09	0.033	0.093
Inter day assay	20.0	19.95 ± 0.15	0.78	0.069	0.192
	50.0	49.95 ± 0.09	0.19	0.041	0.116
	80.0	80.01 ± 0.16	0.20	0.071	0.198

[a] Mean for 5 independent analyses.
[b] SAE, standard analytical error.
[c] C.L., confidence limit at 95% confidence level and 4 degrees of freedom ($t = 2.776$).

regression of the standard addition method or by the extrapolation of the same line of best fit (**Figs. 2.2 and 2.3, Table 2.5**). It is evident from **Table 2.5** that the linearity of the regression line of the standard addition method was good. The attractive feature of the method is its relative freedom from interference by the usual tablet diluents and excipients in amounts far in excess of their normal occurrence in pharmaceutical preparations.

Applicability of the proposed kinetic method

The proposed kinetic method has been successfully applied to the determination of pantoprazole in pharmaceutical preparations. The results obtained by the proposed method were compared to those of the reference method [11] using point and interval hypothesis tests. The results (**Table 2.6**) show that the Student's *t*- and *F*- values at 95% confidence level are less than the theoretical values, which confirmed that there is no significant difference between the performance of the proposed kinetic method and the reference method. The interval hypothesis test has also confirmed that no significant difference exists between the performances of the methods compared, as the true bias of all drug samples is $< \pm 2.0$ %.

The performance of the proposed kinetic spectrophotometric method was compared with other existing conventional spectrophotometric methods (**Table 2.7**). It is apparent from **Table 2.7** that the present procedure requires 4 min only for obtaining the initial rate of reaction, which is proportional to the concentration of pantoprazole. The reaction of pantoprazole with ammonium ferric sulphate and potassium ferricyanide requires 30 min to develop a stable colour, which is mandatory for conventional spectrophotometric methods. Thus, the time of analysis is reduced when the kinetic

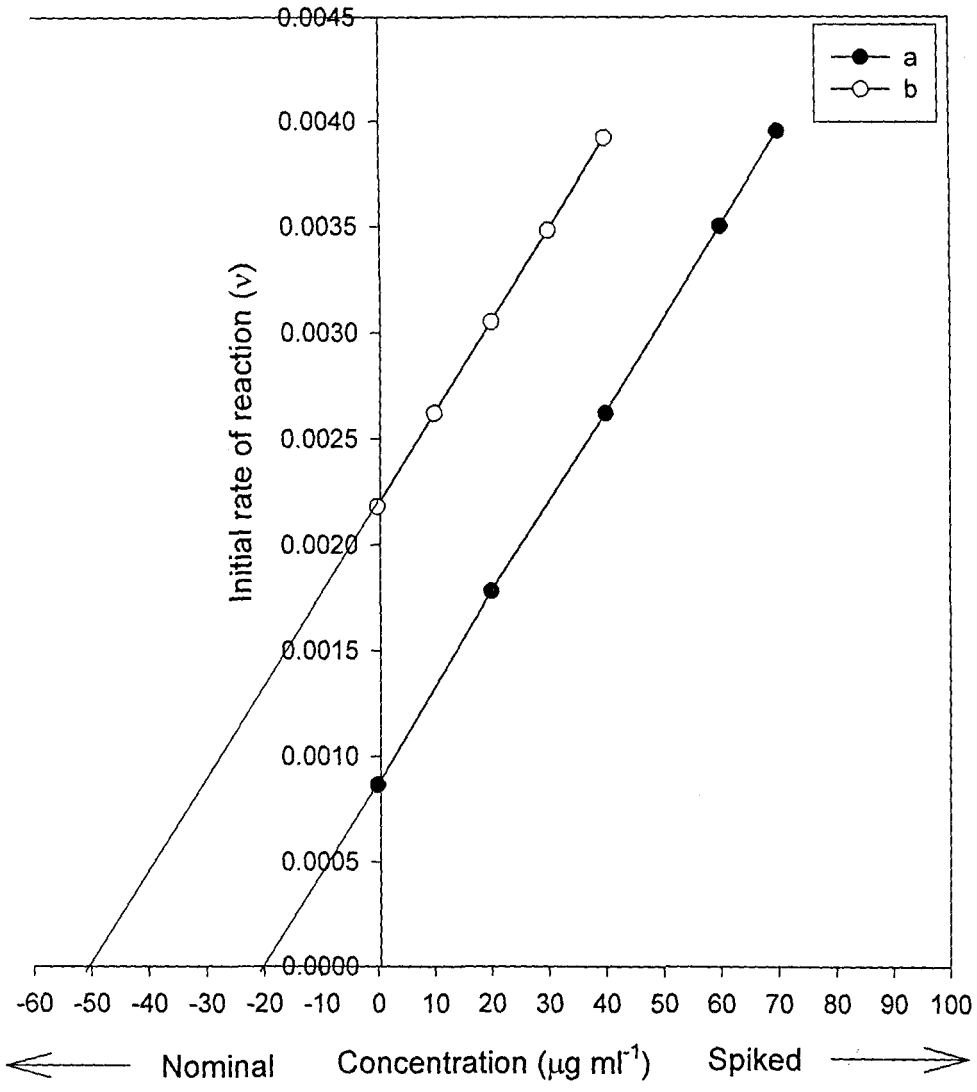


Fig. 2.2. Plot for the recovery evaluation of pantec-20 through standard addition method: (a) 20 and (b) 50 μg ml^{-1}.

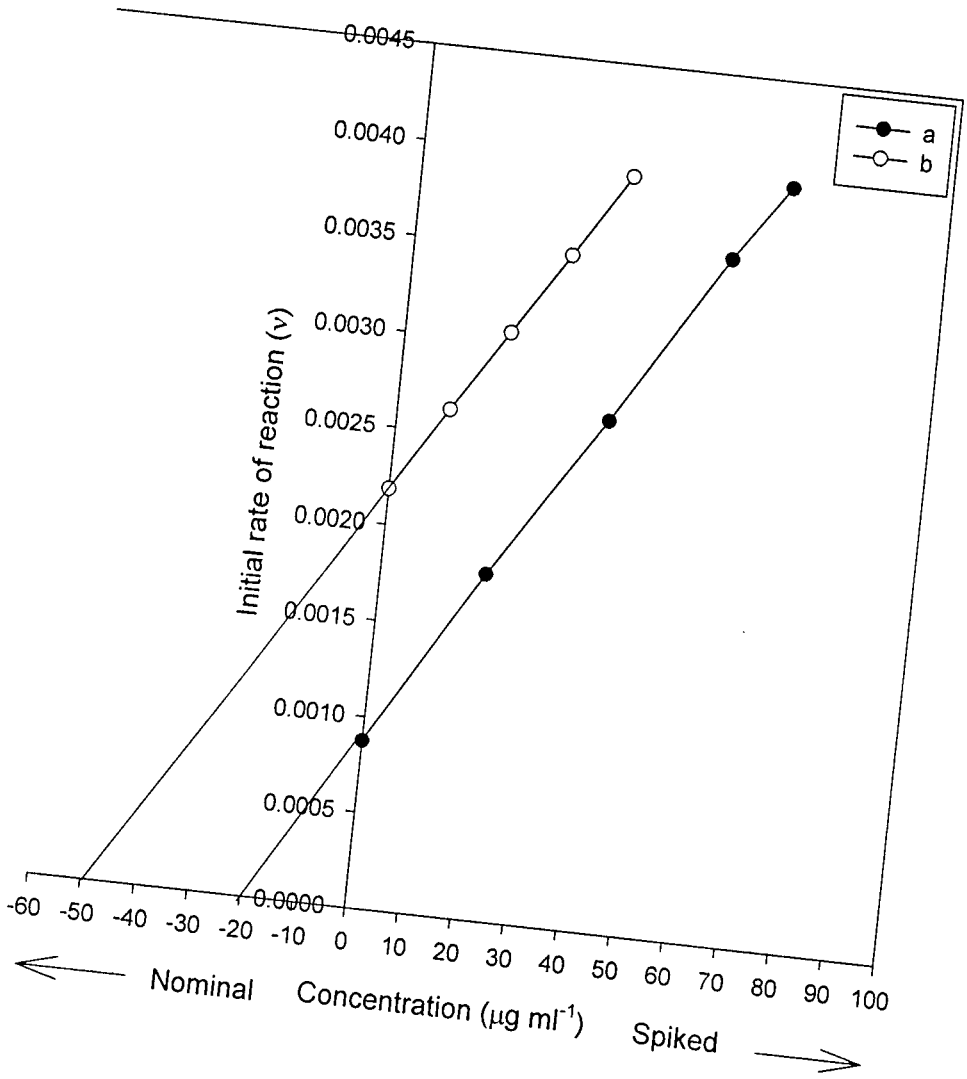


Fig. 2.3. Plot for the recovery evaluation of pantodac-20 through standard addition method: (a) 20 and (b) 50 μg ml^{-1}.

Table 2.5
Test of accuracy for analysis of pantoprazole in drug formulations by standard addition method

Formulations	Concentration ($\mu g\ ml^{-1}$)			Coefficients of linear regression equation of standard addition			Recovery (%)
	Theoretical	Spiked	Nominal	Intercept	slope	r^a	
Pantec-20	20.0	0, 20, 40, 60, 70	20.004	8.766×10^{-4}	4.383×10^{-5}	0.9999	100.02
(Concept)	50.0	0, 10, 20, 30, 40	50.242	2.180×10^{-3}	4.339×10^{-5}	0.9994	100.48
Pantodac-20	20.0	0, 20, 40, 60, 70	20.357	8.729×10^{-4}	4.365×10^{-5}	0.9998	99.99
(Zydus Cadila)	50.0	0, 10, 20, 30, 40	50.233	2.150×10^{-3}	4.280×10^{-5}	0.9998	100.46

[a] Coefficient of correlation.

Table 2.6
Analysis of pantoprazole in commercial dosage forms by proposed method and reference method at 95% confidence level

Formulations	Proposed method		Reference method		Paired t-value[b]	F-value[b]	θ_L[c]	θ_U[c]
	Recovery (%)	RSD[a], (%)	Recovery (%)	RSD[a] (%)				
Pantec-20 (Concept)	99.99	0.10	100.05	0.10	0.114	1.038	0.989	1.009
Pantodac-20 (Zydus Cadila)	100.06	0.10	99.98	0.10	0.279	1.201	0.991	1.011

[a]Mean for 5 independent analyses.
[b]Theoretical *t*-value ($\nu = 8$) and *F*-value ($\nu = 4, 4$) at 95 % confidence level are 2.306 and 6.39, respectively.
[c]In pharmaceutical analysis, a bias, based on recovery experiments, of ± 2% is acceptable.

Table 2.7
Comparison of the proposed kinetic spectrophotometric method with existing conventional spectrophotometric methods for the assay of pantoprazole in pharmaceutical formulations

Reagents	λ_{max} (nm)	Reaction time	Linear dynamic range ($\mu g\ ml^{-1}$)	RSD (%)	Reference
$FeCl_3$ in ethanol	455	30 min at 60° C	30 - 300	1.52	10
2,3-Dichloro-5,6-dicyano-1,4-benzoquinone in acetonitrile	457	Immediately at 25° C	10 - 60	0.53	11
Iodine in chloroform	359	5 min at 25° C	17.7 - 141.6	1.21	11
Copper and eosin in chloroform	549	25 min at 70° C	4.3 - 25.9	0.81	11
Potassium ferricyanide and ammonium ferric sulphate	725	4 min at 35° C	5 - 90	0.78	This work

spectrophotometric procedure was applied. The other conventional spectrophotometric methods using $FeCl_3$ [10] and eosin and copper [11] need longer times to record the absorbance, whereas the method utilizing 2,3-dichloro-5,6-dicyano-1,4-benzoquinone [11] gives a stable colour instantaneously. The drawback of these methods is that they employ an organic solvent while the proposed kinetic method is carried in an aqueous system.

CONCLUSIONS

The proposed kinetic method is a selective one as the drug contains sulphoxide group, which preferentially reduces Fe(III) to Fe(II); subsequently Fe(II) reacts with potassium ferricyanide to form Prussian blue. In human and animals, pantoprazole is metabolized to pantoprazole sulphone, which did not give a positive result with the reagents used. This is a remarkable advantage of the method that Fe(III) in the presence of potassium ferricyanide selectively reacts with pantoprazole and give Prussian blue product. Point and interval hypothesis tests clearly proved that the proposed method has acceptable recovery with a bias of less than ± 2%. The method is also useful due to its wide linear dynamic range (5.0 - 90.0 $\mu g\ ml^{-1}$) and high tolerance limit for common excipients found in drug formulations. Hence, these advantages encourage the application of the proposed kinetic method in routine quality control analysis of pantoprazole in industries, research laboratories and hospitals.

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CHAPTER-3

KINETIC SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF LANSOPRAZOLE IN PHARMACEUTICAL FORMULATIONS

INTRODUCTION

Lansoprazole is an important proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the gastric H^+, K^+ ATPase enzyme system at the secretory surface of the gastric parietal cells [1]. As chemically defined in the Martindale, The Extra Pharmacopoeia, [2] it is 2-[[[3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridinyl]methyl]sulfinyl], 1H-Benzimidazole [103577-45-3; M.W. 369.36]. The drug is effectively useful in the treatment of duodenal ulcer, gastric ulcer, reflux oesophagitis and helicobactor pylori infection. In addition to its efficacy in healing or maintenance treatment it may provide more effective system relief than other comparator agents. Owing to the vital importance of the drug the development of a sensitive, simple and fast method for the determination of the drug is of urgent need.

The assay of drug is only listed in the monograph of The United States Pharmacopoeia, which describes a high performance liquid chromatographic method [3]. Lansoprazole has been determined in pharmaceutical preparations using high performance liquid chromatography [4-6], high performance thin layer chromatography [7,8], capillary electrophoresis [9], flow injection analysis [10] and electroanalytical methods [11,12]. The spectrophotometric methods are the instrumental method of choice and have practical and significant advantages over other methods. In the literature few spectrophotometric methods have been reported [13,14] for the quantification of the cited drug. Moustafa has reported [15] three spectrophotometric methods. First two methods are based on the charge transfer complexation reaction of the drug with 2,3-dichloro 5,6-dicyano 1,4-benzoquinone and iodine. Third method involves the formation of a ternary complex of the drug with eosin and Cu (II), which absorbs maximally at 549 nm. Lansoprazole forms

ion-pair complex with bromocresol green [16], which was extracted into organic solvent for quantitative determination at 420 nm. The spectrophotometric procedures for its determination in commercial dosage forms have also been discussed based on the reaction with p-dimethylaminobenzaldehyde [17], folin-ciocaltaeu's phenol reagent [18] and vanillin [19].

The literature is still lacking a kinetic spectrophotometric method for the determination of cited drug. Furthermore some specific advantages in the application of kinetic method can be expected such as selectivity due to measurement of the evolution of the absorbance with the time of the reaction.

In this chapter, a kinetically based spectrophotometric method is proposed for the determination of lansoprazole in bulk and capsules by measuring the change in the absorbance at 610 nm and 530 nm followed by oxidation of drug with alkaline $KMnO_4$ at 25 ± 1^0C. The proposed methods are validated statistically as accuracy and reliability of the analytical results is crucial for ensuring quality, safety and efficacy of the pharmaceuticals.

EXPERIMENTAL

Apparatus

A Shimadzu UV-visible spectrophotometer (Model no.1601, Kyoto, Tokyo, Japan) with matched quartz cells was used to measure the absorption spectra and Spectronic 20 D^+ (Milton Roy Company, USA) for recording the absorbance of the solution.

Reagents

All reagents used were of analytical and pharmaceutical grade. Potassium permanganate, 6.0 × 10^{-3}M (GR Grade, Merck Limited, Mumbai, India) solution

should be freshly prepared and its apparent purity was assayed titrimetrically [20]. 1.0 M Sodium hydroxide (E. Merck, India) solution was prepared in distilled water.

Test solution

Lansoprazole (Cipla, India Ltd.), 0.1%(w/v), solution was prepared by dissolving 100 mg in 0.5 ml of 0.10M NaOH and then diluted to 100 ml with distilled water. Lansoprazole solution was freshly prepared and used within 5 hours.

Marketed products

The commercial pharmaceutical preparations of lansoprazole such as Lanzol (Cipla Ltd., Mumbai, India), Lansofast (Cadila Healthcare Ltd., Ahemdabad, India), Propilan (Glenmark Pharmaceuticals Ltd., Mumbai, India) and Lancid (Brown and Burk Pharmaceuticals Ltd., Bangalore, India) were purchased from local drug store.

Recommended Procedure

Method A

Aliquots of 0.1% lansoprazole solution (5 – 150 μg ml^{-1}) were pipetted into a series of 10 ml volumetric flask, after that 1.5 ml of 1M NaOH was added followed by 2.0 ml of 6.0 × 10^{-3}M potassium permanganate solution and then diluted to volume with distilled water. The contents of the flask were mixed well and immediately transferred to the spectrophotometric cell. The absorbance was recorded as a function of time at 610 nm against the reagent blank prepared simultaneously at 25 ± 1°C. The initial rate of formation of MnO_4^{2-} at different concentration was evaluated from the slope of the tangent to the absorbance-time curve. The calibration curve was constructed by plotting initial rate of reaction against the final concentration of lansoprazole in μg ml^{-1}. The amount of lansoprazole was calculated either from the calibration curve or corresponding regression equation.

Method B

Aliquots of 0.1% lansoprazole solution corresponding to 50 - 700 µg were transferred into a series of 10 ml volumetric flask. Then 1 ml of 1M NaOH was added followed by 0.8 ml of 6.0×10^{-3} M potassium permanganate solution and completed the volume with distilled water. The decrease in absorbance of the solution was measured as a function of time at 530 nm. The initial rate of disappearance of MnO_4^- was determined from the slope of tangent to the absorbance-time curve. The calibration curve was constructed by plotting ΔA vs. lansoprazole concentration. The amount of lansoprazole was calculated either from the calibration graph or corresponding regression equation.

Procedure for determination of Lansoprazole in capsules

The content of the capsules (enteric coated granules) were accurately weighed and finely powdered. The amount of the powder equivalent to 200 mg of lansoprazole was dissolved in 50 ml of methanol and allowed to stand for few minutes and filtered on Whatmann No.42 filter paper. The filtrate was evaporated to dryness and the residue was dissolved in 0.5 ml of 0.1M NaOH and diluted to 25 ml with distilled water. The recovery of lansoprazole was calculated from the corresponding linear regression equations or calibration graphs.

RESULTS AND DISCUSSION

The absorption spectrum of lansoprazole solution in distilled water shows two absorption bands peaking at 197 and 287.5 nm while that of potassium permanganate solution in alkaline medium exhibits an absorption band peaking at 530 nm. The course of the reaction starts on the addition of aqueous alkaline potassium permanganate to the solution of lansoprazole resulting in the formation of

new band peaking at 610 nm (**Fig. 3. 1 a, b, c**). This band is attributed due to the formation of manganate ion in the presence of drug. Thus, the intensity of the green coloured solution increases with time owing to the formation of MnO_4^{2-} whereas the absorbance of the solution measured at 530 nm decreases as the reaction proceeds due to the disappearance of MnO_4^-. This was used to develop a kinetically based spectrophotometric method for the determination of lansoprazole.

The optimum conditions affecting the formation of manganate ion were studied and maintained throughout the experiment.

The effect of $KMnO_4$ concentration on the absorbance was studied in the range $1.2 \times 10^{-4} - 1.44 \times 10^{-3}$ M (**Fig. 3.2**) keeping all other experimental parameters constant, [lansoprazole] = 100.0 μg ml^{-1} and [NaOH] = 0.1M. It can be seen that the highest absorbance was obtained at 9.6×10^{-4} M, above this concentration up to 1.44×10^{-3} M, the absorbance was unchanged. Thus, the adoption of 1.2×10^{-3} M $KMnO_4$ in the final solution proved to be sufficient for the maximum concentration of lansoprazole used in the determination process.

The dependence of NaOH concentration on the maximum absorbance was studied in the range 0.02 - 0.2 M. **Fig. 3.3** shows that the absorbance increases with increasing NaOH concentration and maximum absorbance was obtained with 0.09M, after that further increase in the concentration of NaOH up to 0.2 M resulted in no change in absorbance. Hence, 0.15 M NaOH was recommended for determination procedure.

In method A, under the optimized experimental conditions, the determination of lansoprazole was carried out in excess of potassium permanganate and sodium hydroxide solutions with respect to the initial concentration of lansoprazole. As a result pseudo zero order reaction condition was obtained with respect to their

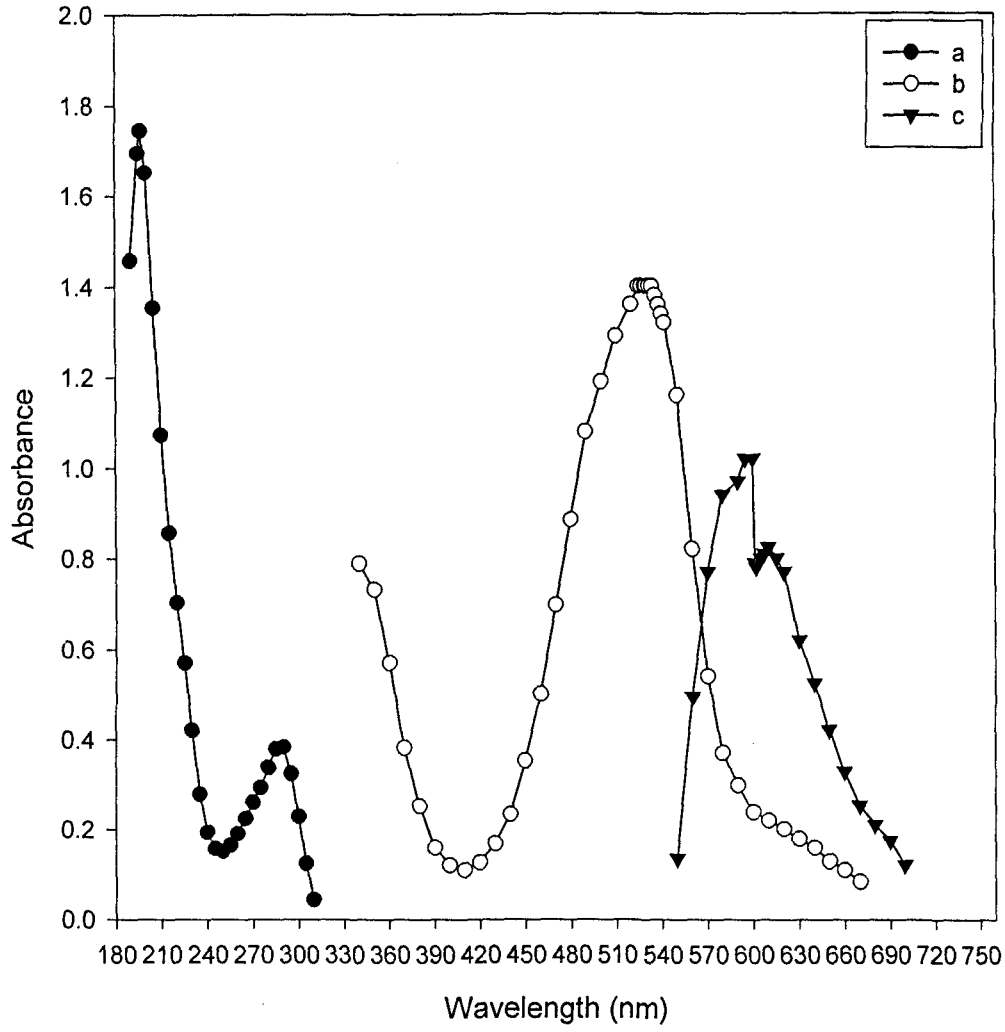

Fig. 3.1. Absorption spectra of (a) 1.0 ml of 2.707×10^{-4} M lansoprazole in distilled water (b) 1.0 ml of 4.50×10^{-3} M $KMnO_4$ + 1.5 ml of 1.0 M NaOH solutions in distilled water and (c) 1.0 ml of 2.707×10^{-3} M lansoprazole + 2.0 ml of 6.00×10^{-3} M $KMnO_4$ and 1.5 ml of 1.0 M NaOH solutions in distilled water. Each set is diluted in 10 ml standard flask with distilled water.

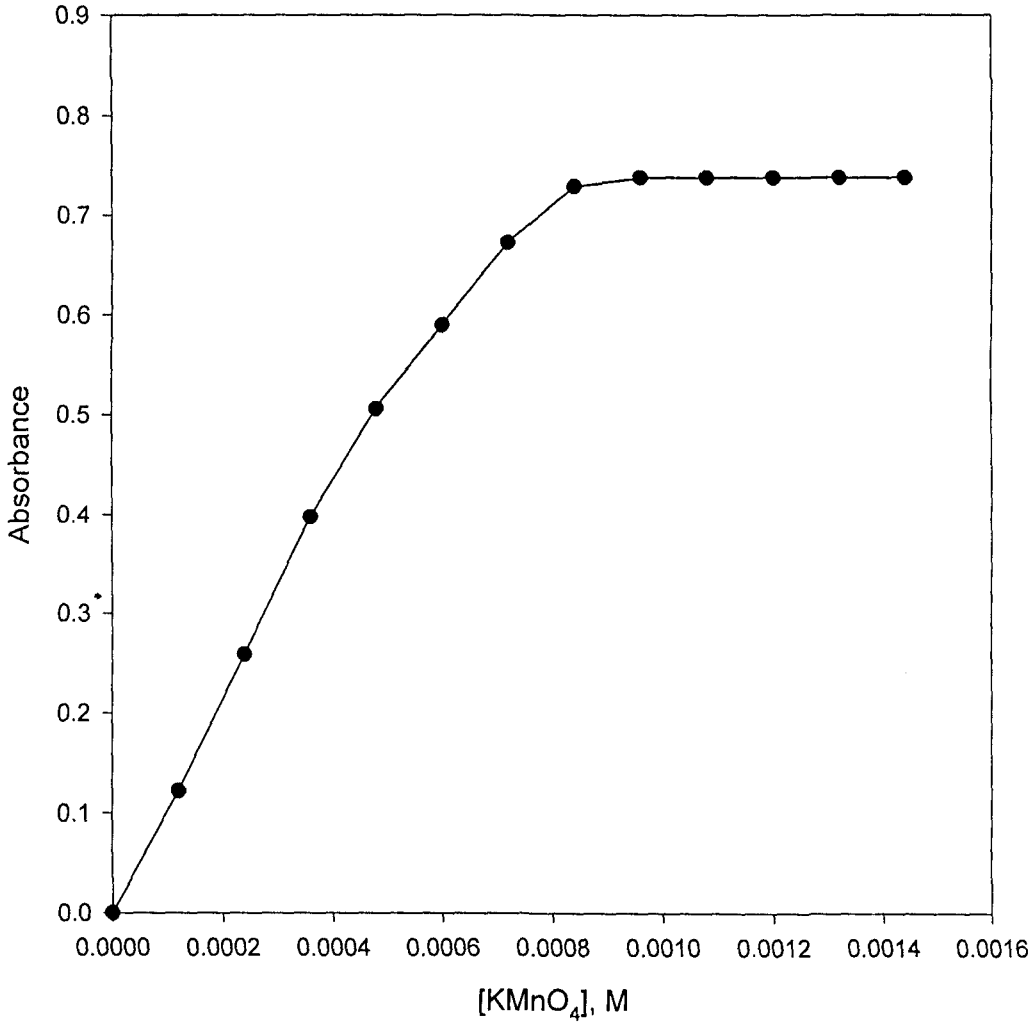

Fig. 3.2. Effect of the concentration of $KMnO_4$ on the absorbance at 610 nm (100.0 μg ml^{-1} lansoprazole).

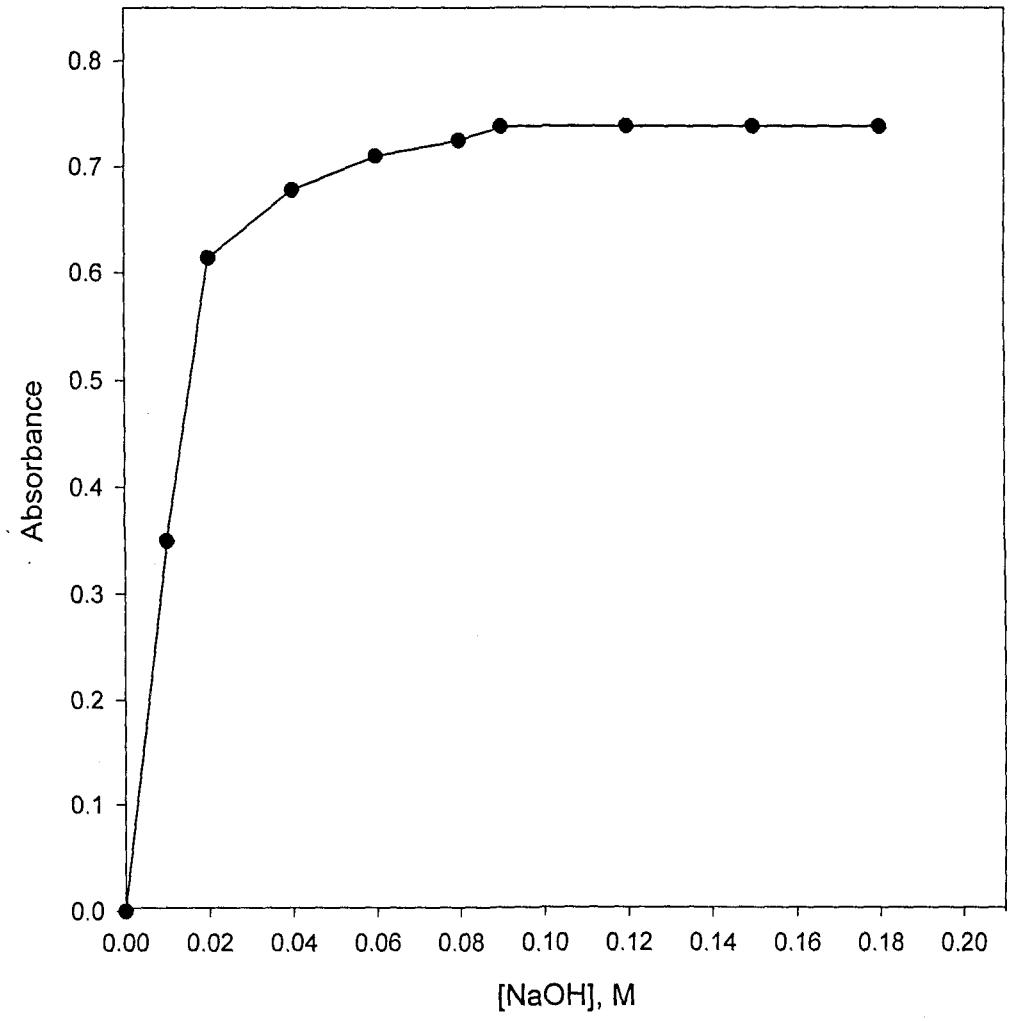

Fig. 3.3. Effect of the concentration of NaOH on the absorbance at 610 nm (100.0 μg ml^{-1} lansoprazole).

concentrations. Therefore, on the basis of kinetic investigation, kinetic equation for the oxidation of lansoprazole by $KMnO_4$ in alkaline medium is written as:

$$\text{rate} = k\,[C]^n\,[KMnO_4]^m\,[NaOH]^l \quad (1)$$

For $[KMnO_4] \geq 1.20 \times 10^{-3}$ M and $[NaOH] \geq 0.15$ M

The equation (1) reduces to:

$$\text{rate} = k_\psi\,[C]^n \quad (2)$$

where K_ψ is the pseudo-order rate constant, C is the concentration of lansoprazole and n is the order of reaction. The logarithmic form of equation (2) may be written as:

$$\log(\text{rate}) = \log K_\psi + n \log C \quad (3)$$

The initial rates of reaction were evaluated at different concentrations of lansoprazole by measuring the slopes of the initial tangent to the absorbance (at 610 nm) - time curves during the first 10 min of the reaction (**Fig. 3.4**) and the values of the results are summarized in **Table 3.1**. The curve of log rate versus log C gave the following linear equation:

$$\log(\text{rate}) = 1.281 + 0.998 \log C$$

with a correlation coefficient (r) of 0.9998. The value of n in the equation confirmed that the reaction is first order with respect to lansoprazole and rate constant is 19.1 s^{-1}.

The calibration curve was prepared by plotting initial rate versus concentration of lansoprazole and found to be linear over the concentration range of 5 - 150 μg ml^{-1}. The regression analysis of calibration data yielded the following regression equation:

$$\text{rate} = -3.915 \times 10^{-6} + 5.271 \times 10^{-5}\,C$$

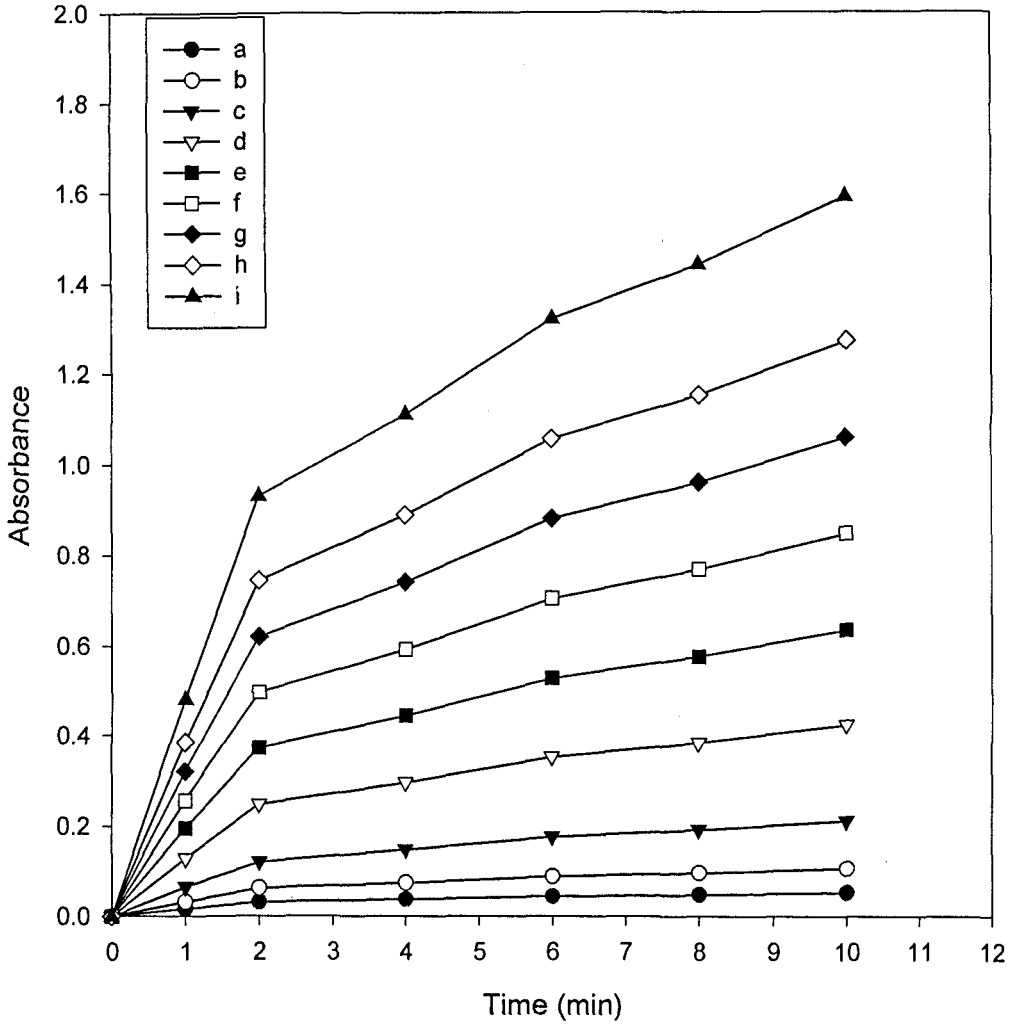

Fig. 3.4. Absorbance (at 610 nm)-time curves for the first 10 min of the reaction between lansoprazole and $KMnO_4$ in distilled water: 2.0 ml of 0.006 M $KMnO_4$ and lansoprazole: (a) 5, (b) 10, (c) 20, (d) 40, (e) 60, (f) 80, (g) 100, (h) 120 and (i) 150 μg ml^{-1}. Each set is diluted in 10 ml standard flask with doubly distilled water.

Table 3.1
Initial rate of reaction (formation of MnO_4^{2-}) at different concentration of lansoprazole

C, [Drug] mol L^{-1}	log C	Initial rate of reaction, υ mol l^{-1} s^{-1}	log υ
1.353×10^{-5}	-4.868	2.583×10^{-4}	-3.587
2.707×10^{-5}	-4.567	5.500×10^{-4}	-3.259
5.414×10^{-5}	-4.266	1.055×10^{-3}	-2.976
1.083×10^{-4}	-3.965	2.083×10^{-3}	-2.681
1.624×10^{-4}	-3.789	3.166×10^{-3}	-2.499
2.166×10^{-4}	-3.664	4.166×10^{-3}	-2.380
2.707×10^{-4}	-3.567	5.300×10^{-3}	-2.275
3.248×10^{-4}	-3.488	6.283×10^{-3}	-2.201
4.061×10^{-4}	-3.391	7.933×10^{-3}	-2.100

(n =9, ± tSa = 4.279 × 10^{-5}, ± tS$_b$ = 5.285 × 10^{-4} at 95% confidence level) with coefficient of correlation, r = 0.9999. The variance was calculated [21] using statistical treatment of calibration data at nine concentration levels and found to be 9.80 × 10^{-10} μg ml^{-1}. The low value of variance speaks the negligible scattering of the experimental data points around the line of regression.

In method B, the oxidation of lansoprazole by potassium permanganate in alkaline medium was followed spectrophotometrically by measuring the decrease in absorbance at 530 nm against a reagent blank prepared similarly except drug. The absorbance-time curves are shown in **Fig. 3.5**. The difference between the absorbance obtained at 2 min and the absorbance at 4, 6, 8 and 10 min was plotted against the concentration of lansoprazole and the corresponding linear regression with coefficient of correlation is summarized in **Table 3.2**. It is clear from the table that the most acceptable linearity was obtained at A_2 - A_8, therefore, the quantitative analyses of lansoprazole in each sample was carried out at A_2 - A_8. The calibration curve was linear in the range of 5 - 70 μg ml^{-1} of lansoprazole and the corresponding regression equation is $\Delta A = 1.04 \times 10^{-3} + 1.78 \times 10^{-3}$ C (n = 8, r = 0.9991, ± tSa = 3.132 × 10^{-3}, ± tS$_b$ = 7.457 × 10^{-5} at 95% confidence level). It was found that the variance of this procedure was 3.61 × 10^{-6} μg ml^{-1}.

The absolute error (S_x) in the determination of a given concentration of lansoprazole has been evaluated [22] using the following equation:

$$S_x = \frac{S_0}{b}\left[1 + \frac{1}{n} + \frac{(y-\bar{y})^2}{b^2\sum_i (x_i - \bar{x})^2}\right]^{1/2}$$

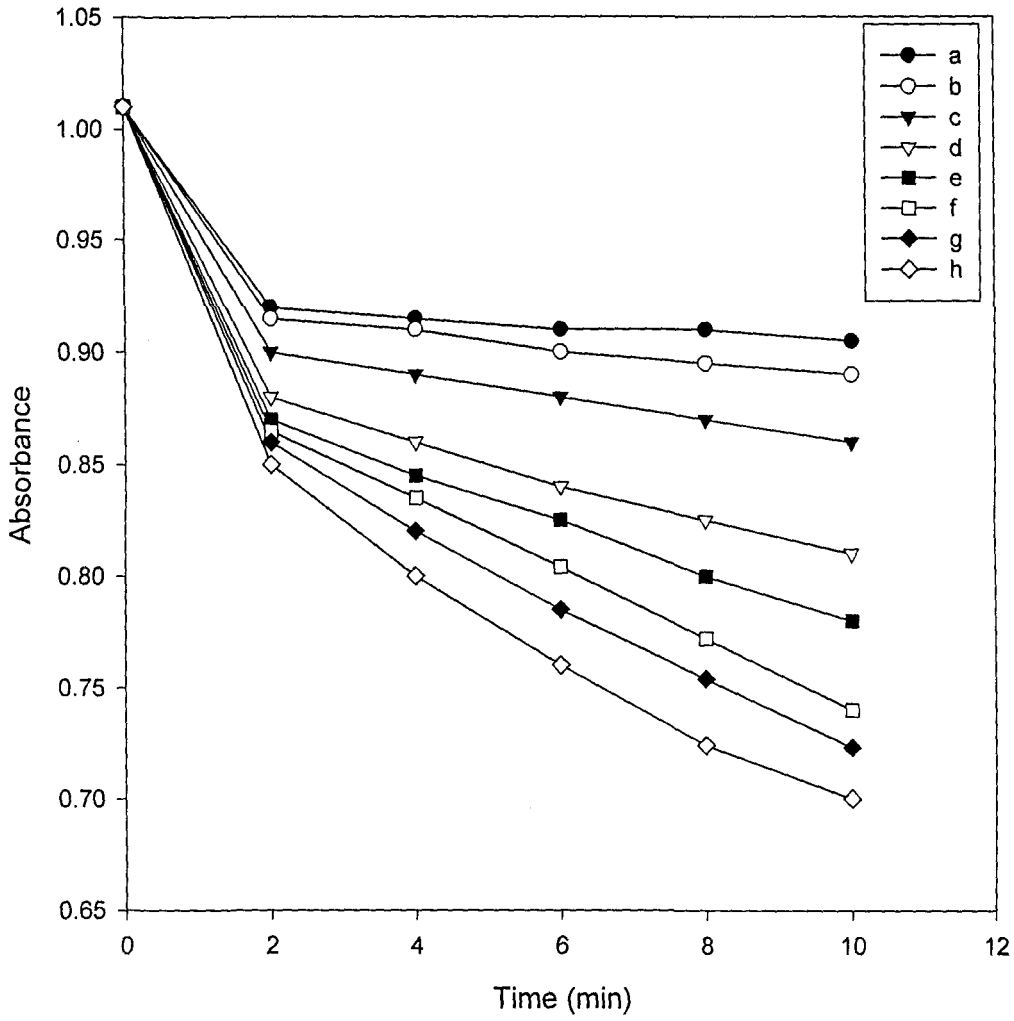

Fig. 3.5. Absorbance (at 530 nm)-time curves for the first 10 min of the reaction between lansoprazole and $KMnO_4$ in distilled water: 0.8 ml of 0.006 M $KMnO_4$ and lansoprazole: (a) 5, (b) 10, (c) 20, (d) 30, (e) 40, (f) 50, (g) 60, and (h) 70 μg ml^{-1}. Each set is diluted in 10 ml standard flask with doubly distilled water.

Table 3.2
Calibration equations with coefficient of correlation (r) at A_2 - A_4, A_2 - A_6, A_2 - A_8 and A_2 - A_{10} for method B

ΔA	Calibration equation	r
A_2 - A_4	$A = -2.300 \times 10^{-4} + 6.731 \times 10^{-4} C$	0.9907
A_2 - A_6	$A = 1.980 \times 10^{-3} + 1.210 \times 10^{-3} C$	0.9955
A_2 - A_8	$A = 1.040 \times 10^{-3} + 1.780 \times 10^{-3} C$	0.9991
A_2 - A_{10}	$A = 3.130 \times 10^{-3} + 2.200 \times 10^{-3} C$	0.9942

and was plotted in **Figs. 3.6** and **3.7.** It is found from the figures that the minimum error was obtained when the analysis was done at 65.0 and 35.6 μg ml^{-1} using methods A and B, respectively. The value of S_x also allows to establish the confidence limit at the selected level of significance for the determination of unknown concentrations by using the relation, $Ci \pm t_p S_x$.

The accuracy and precision of the proposed procedures (Methods A and B) was established by measuring the content of lansoprazole at three different concentration levels (low, medium and high) within one day and on five consecutive days. The intra day and inter day assays were performed by measuring six independent analyses at 10.0, 60.0 and 130.0 μg ml^{-1} concentration levels using method A. The same assays (intra and inter day) were too done for method B at concentration levels: 10.0, 30.0 and 60.0 μg ml^{-1} on one day as well as on five consecutive days. The results of standard deviation, relative standard deviation and mean recoveries obtained by intra day and inter day assays for method A and B are acceptable and can be considered to be very satisfactory (**Table 3.3**).

As an additional demonstration of accuracy, recovery experiments were performed by adding a known amount of lansoprazole to the preanalysed dosage forms. The total amount of lansoprazole was determined by proposed methods and the results are reported in **Table 3.4**. It is evident from the table that mean recoveries were in the range of 99.48% - 100.38% and 99.31% - 100.18% for methods A and B, respectively.

The proposed methods (A and B) were successfully applied to the determination of lansoprazole in capsules. The results of proposed methods were compared with those of the reference method [15] using point and interval hypothesis tests. Calculated *t*- and *F*-values shown in **Table 3.5** are less than the

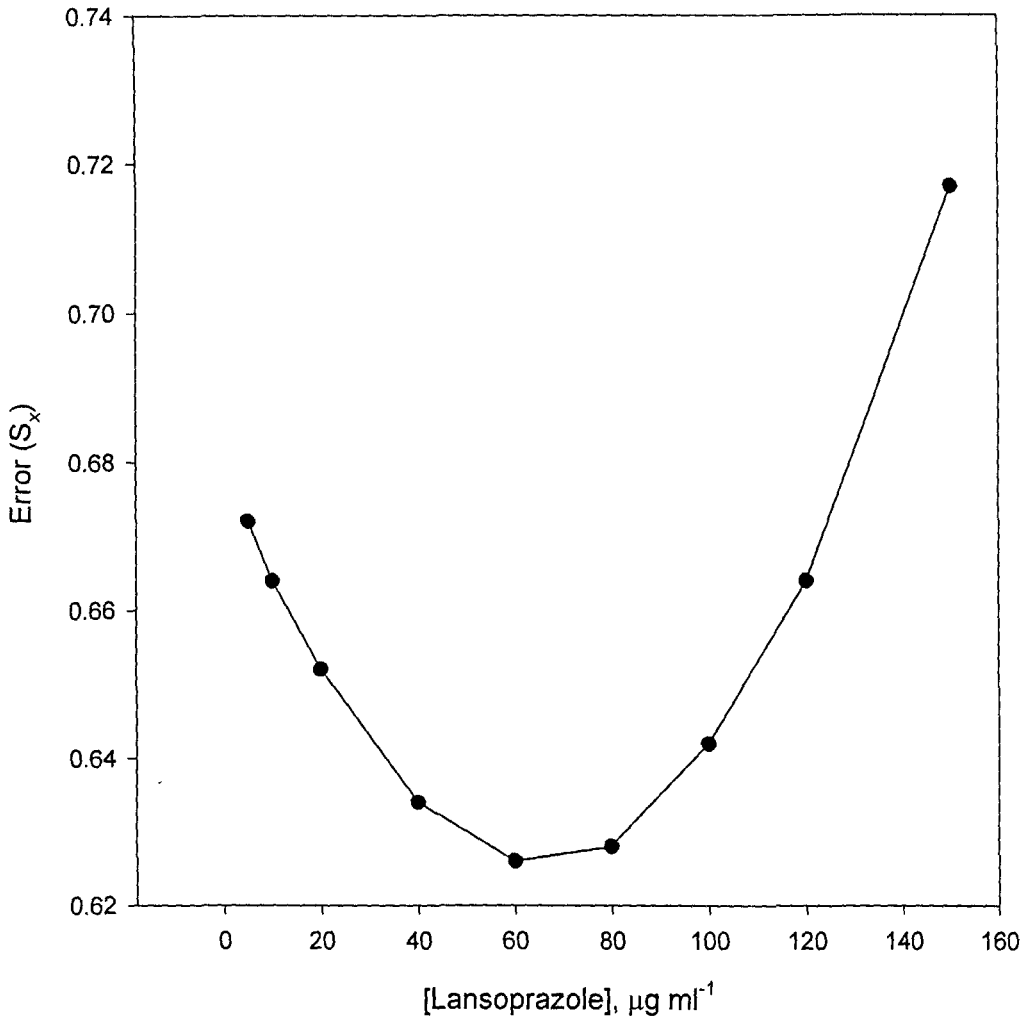

Fig. 3.6. Error (S_x) in the determination of lansoprazole with Method A.

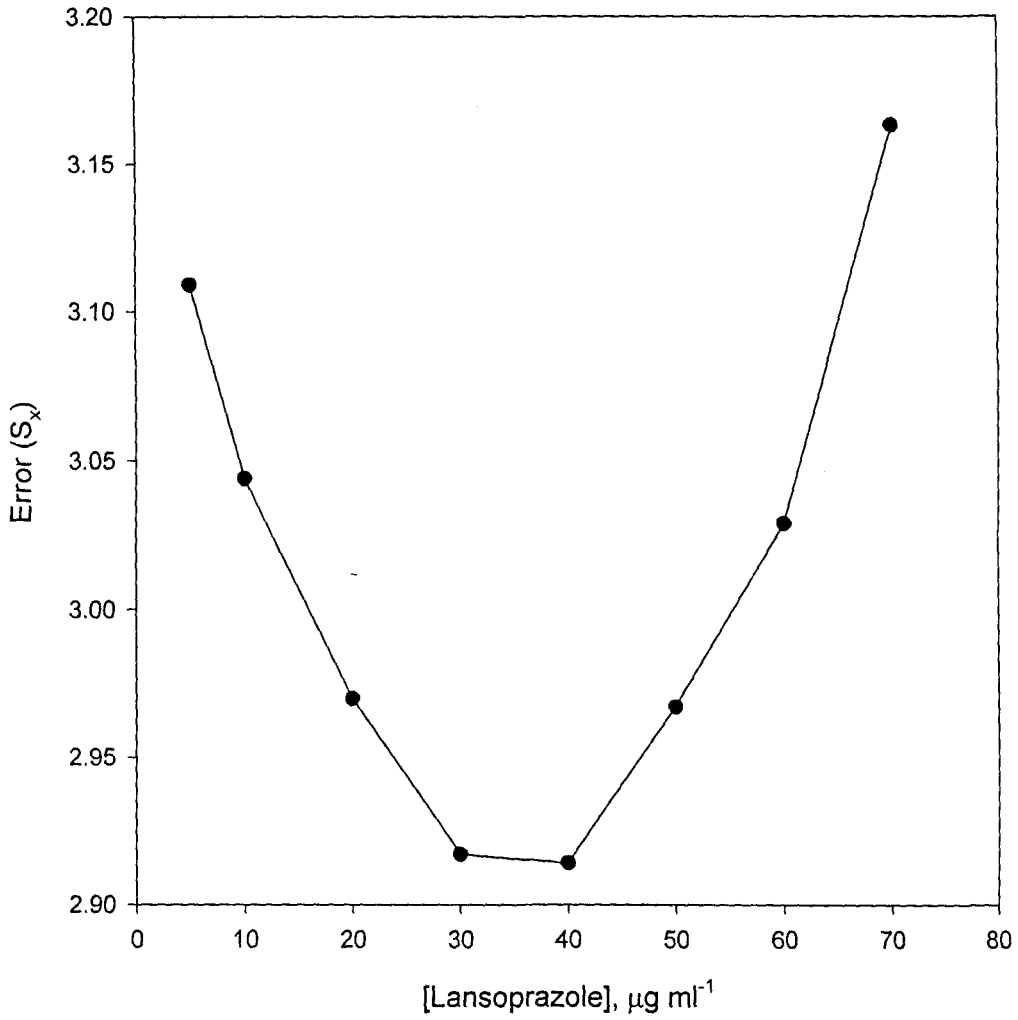

Fig. 3.7. Error (S_x) in the determination of lansoprazole with Method B.

Table 3.3
Intra day and inter day assays: test of precision of methods A and B

Proposed methods	Amount ($\mu g\ ml^{-1}$)		Recovery (%)	RSD (%)	SAE	C.L.
	Taken	Found ± SD[a]				
Method A						
Intra day assay	10.0	9.90 ± 0.17	99.05	1.77	0.069	0.177
	60.0	59.87 ± 0.37	99.79	0.63	0.154	0.394
	130.0	130.11 ± 0.54	100.08	0.42	0.220	0.565
Inter day assay	10.0	9.91 ± 0.10	99.15	1.04	0.042	0.107
	60.0	60.11 ± 0.65	100.11	1.00	0.268	0.689
	130.0	129.30 ± 0.93	99.50	0.72	0.340	0.874
Method B						
Intra day assay	10.0	10.03 ± 0.10	100.31	0.99	0.020	0.051
	30.0	29.97 ± 0.20	99.91	0.67	0.081	0.208
	60.0	60.08 ± 0.40	100.14	0.67	0.164	0.421
Inter day assay	10.0	9.90 ± 0.16	99.02	1.61	0.065	0.167
	30.0	29.98 ± 0.19	99.93	0.66	0.080	0.205
	60.0	59.85 ± 0.57	99.75	0.96	0.234	0.601

[a] Mean for six independent analyses.
[b] SAE, standard analytical error.
[c] C.L., confidence limit at 95 % confidence level and five degrees of freedom (t = 2.571)

Table 3.4

Standard addition method for the determination of lansoprazole in commercial capsules

Formulations	Method A						Method B					
	Amount ($\mu g\ mL^{-1}$)			Recovery (%)	RSD (%)	SAE	Amount ($\mu g\ mL^{-1}$)			Recovery (%)	RSD (%)	SAE
	Taken	Added	Found ± SD[a]				Taken	Added	Found ± SD[a]			
Lanzol 30 (Cipla)	10.0	10.0	20.02 ± 0.28	100.11	1.43	0.117	10.0	10.0	20.03 ± 0.35	100.18	1.78	0.145
	20.0	40.0	59.81 ± 0.61	99.68	1.02	0.250	20.0	20.0	39.94 ± 0.29	99.85	0.75	0.121
	50.0	80.0	130.09 ± 0.71	100.07	0.54	0.289	30.0	30.0	59.71 ± 0.59	99.51	1.00	0.244
Lansofast 30 (Cadila)	10.0	10.0	20.01 ± 0.35	100.01	0.54	0.290	10.0	10.0	19.87 ± 0.28	99.35	1.49	0.114
	20.0	40.0	60.03 ± 0.37	100.05	0.61	0.151	20.0	20.0	39.95 ± 0.34	99.88	0.86	0.140
	50.0	80.0	129.74 ± 0.49	99.80	1.11	0.591	30.0	30.0	60.11 ± 0.29	100.18	0.49	0.120
Propilan 30 (Glenmark)	10.0	10.0	19.90 ± 0.27	99.53	1.35	0.110	10.0	10.0	19.86 ± 0.27	99.31	1.40	0.111
	20.0	40.0	60.08 ± 0.31	100.14	0.51	0.126	20.0	20.0	39.93 ± 0.32	99.82	0.78	0.128
	50.0	80.0	129.33 ± 0.92	99.48	0.71	0.378	30.0	30.0	59.82 ± 0.57	99.71	0.96	0.235
Lancid 30 (Brown & Burk)	10.0	10.0	20.00 ± 0.29	100.02	1.49	0.122	10.0	10.0	19.99 ± 0.28	99.95	1.40	0.114
	20.0	40.0	59.86 ± 0.25	99.76	0.42	0.104	20.0	20.0	39.94 ± 0.31	99.83	0.78	0.128
	50.0	80.0	130.50 ± 0.83	100.38	0.64	0.338	30.0	30.0	60.08 ± 0.41	100.14	0.67	0.164

[a]Mean for six independent analyses.

Table 3.5
Point and interval hypothesis tests: comparison of the proposed methods with the reference method at 95% confidence level

Formulations	Method A						Method B						Reference method	
	Recovery (%)	RSD[a] (%)	t-value[b]	F-value[b]	θ_L[c]	θ_U[c]	Recovery (%)	RSD[a] (%)	t-value[b]	F-value[b]	θ_L[c]	θ_U[c]	Recovery (%)	RSD[a] (%)
Lanzol 30 (Cipla)	99.86	0.46	0.35	1.20	0.995	1.006	99.98	0.65	0.65	1.19	0.995	1.008	99.76	0.55
Lansofast 30 (Cadila)	99.76	0.47	0.95	1.29	0.991	1.002	99.91	0.79	0.37	1.28	0.991	1.005	100.06	0.62
Propilan 30 (Glenmark)	100. 09	0.54	0.81	1.18	0.996	1.009	100.11	0.42	0.97	1.55	0.997	1.008	99.81	0.64
Lancid 30 (Brown & Burk)	100.19	0.51	0.97	1.03	0.997	1.007	99.78	0.61	0.39	1.26	0.992	1.004	99.91	0.48

[a] Mean for six independent analyses.
[b] Theoretical t-value (ν= 10) and F-value (ν= 5, 5) at 95 % confidence level are 2.228 and 5.05, respectively.
[c] In pharmaceutical analysis, a bias, based on recovery experiments, of ±2% (θ_L= 0.98 and θ_U = 1.02) is acceptable.

theoretical ones [23], confirming accuracy and precision at 95% confidence level. The interval hypothesis test [24] has also confirmed that true bias of all samples is smaller than ± 2% at 95% confidence level, since the acceptable interval is within $\theta_L = 0.98$ and $\theta_U = 1.02$.

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CHAPTER-4

SPECTROPHOTOMETRIC DETERMINATION OF ESOMEPRAZOLE MAGNESIUM IN COMMERCIAL TABLETS USING 5-SULPHOSALICYLIC ACID AND N-BROMOSUCCINIMIDE

INTRODUCTION

Esomeprazole magnesium is chemically known as 5-methoxy-2- {(S)-[(4-methoxy-3, 5-dimethyl-2-pyridyl) methyl] sulfinyl} benzimidazole magnesium trihydrate [1,2]. This is the first proton pump inhibitor developed as a single optical isomer for the treatment of acid-related diseases. It has the advantages over omeprazole in terms of pharmacokinetic characteristics and acid suppression. The drug is used in the management of patients with gastroesophageal reflux disease, erosive reflux esophagitis and peptic ulcer. The drug is a weak base that is concentrated in the acidic compartment of secretory canaliculus of the parietal cell where it undergoes acid-catalysed transformation to a tetracyclic achiral cationic sulphenamide. This then reacts with specific cysteines resulting in the inhibitation of the H^+ / K^+- ATPase enzyme [3]. The drug is officially listed in Martindale The Extra Pharmacopoeia [1]. As far as we are aware that the assay of drug in bulk and dosage forms is not cited in any pharmacopoeia (USP or BP or Ph Eur or IP) and literature is also not available for its determination. Therefore, there is an urgent need for the development of simple, sensitive and accurate methods for the determination of esomeprazole magnesium in pharmaceutical products.

This chapter describes two rapid, simple, sensitive and economical spectrophotometeric methods for the determination of esomeprazole magnesium in commercial dosage forms. Method A is based on the reaction of esomeprazole magnesium with 5 sulphosalicylic acid in methanol leading to the formation of a yellow coloured complex which absorbs maximally at 365 nm, Method B utilizes the reaction of drug with N-bromosuccinimide in acetone-chloroform medium resulting in the formation of yellow coloured α-bromo derivative of the drug. The absorption spectrum shows a band peaking at 380 nm. The proposed methods are

validated as per the guidelines of International Conference on Harmonisation (USA) [4].

EXPERIMENTAL

Apparatus

All absorbance measurements were made on a spectronic $20D^+$ spectrophotometer (Milton Roy Company, USA) with 1cm matched glass cells. The absorption spectra were recorded on a shimadzu uv-visible spectrophotometer (UV-1240, Shimadzu Corporation, Kyoto, Japan) with matched quartz cells.

Material and reagents

All chemicals and reagents were of analytical grade. The reference standard of esomeprazole magnesium (Batch No. BE0-0840009) was kindly provided by Torrent Pharmaceuticals Ltd., Ahmedabad, India. The commercial dosage forms of esomeprazole magnesium such as Nexpro-20 (Torrent Pharmaceuticals Ltd. Ahmdabad, India), Raciper-20 (Ranbaxy Laboratories Ltd., New Delhi, India) and Esoz-20 (Glenmark pharmaceuticals Ltd. Mumbai, India) were obtained from local drug stores. 5-sulphosalicylic acid (Qualigens Fine Chem. Ltd., Mumbai, India) was prepared as 1.97×10^{-3} M solution in methanol. N-bromosuccinimide solution (s.d. fine-chem. Ltd., Mumbai, India) was prepared as 2.25×10^{-2} M in acetone.

Standard solutions

- esomeprazole magnesium (0.2 mg ml^{-1}) solution was prepared in methanol.
- esomeprazole magnesium (0.5 mg ml^{-1}) solution was prepared in chloroform.

Procedure for the determination of esomeprazole magnesium

Method A

Aliquots (0.05 - 1.2) ml of standard solution of esomeprazole magnesium (0.2 mg ml^{-1}) were mixed with 1.2 ml of 1.97×10^{-3} M 5-sulfosaicylic acid into a series of conical flasks and then heated at 50 ± 1°C for 5 min. The contents of the mixture were transferred into 5 ml standard volumetric flask and then diluted up to volume with methanol. The absorbance was recorded at 365 nm within the stability time of 5 h against the reagent blank prepared simultaneously except the drug. The amount of the drug in the given sample can be estimated either from calibration graph or corresponding regression equation.

Method B

The volumes (0.1 - 1.0 ml) of standard solution of esomeprazole magnesium (0.5 mg ml^{-1}) were transferred into a series of conical flasks followed by 2.2 ml of 2.25×10^{-2} M N-bromosuccinimide and warmed at 35 ± 1°C for 12 min. After cooling at room temperature (25 ± 1°C), the contents of each conical flask were transferred to 5 ml standard volumetric flask and then diluted up to the mark with acetone. The absorbance was measured at 380 nm against the reagent blank prepared simultaneously omitting esomeprazole magnesium. The colour of the complex was stable for at least 1 h. The concentration of the drug was calculated either from calibration curve or corresponding regression equation.

Procedure for the assay of esomeprazole magnesium in pharmaceutical formulations

Ten and twenty-five tablets of 20 mg strength of esomeprazole magnesium were taken separately in methanol and chloroform, for methods A and B, respectively and left for 10 min for complete dispersion of the drug. The methanol and chloroform extracts were filtered through Whatmann No. 42 filter paper

(Whatmann International Limited, Kent, UK) in 100 ml volumetric flasks, separately. The residues were washed well with 5 × 10 ml portion of methanol and chloroform, as the case may be, for complete recovery of the drug and finally diluted to volume appropriate solvent. The amount of the drug was determined following the proposed procedures.

Procedure for the reference method

Aliquots of 0.05 - 1.0 ml of standard solution of esomeprazole magnesium (0.2 mg ml^{-1}) were pipetted into a series of 10 ml volumetric flasks and diluting to volume with chloroform. The absorbance was measured at 301.5 nm against the solvent blank. The amount of the drug in the given sample was computed from the calibration graph.

Determination of validation parameters

The proposed methods have been validated for specificity and selectivity, linearity, accuracy and precision, limits of detection and quantitation, and recovery.

Solution stability

The solution stability of pure drug and quality control samples was ascertained by observing UV-visible spectra and spots on TLC plates for several days at room temperature. The thin layer chromatography was performed using TLC plates coated with silica gel G (Merck Limited, Mumbai, India) and developed in dichloromethane-methanol-2.67 M ammonia (5:0.3:2 v/v/v) solvent system. Then the TLC plates were freed from mobile phase, dried and spots were detected in iodine chamber.

Specificity and selectivity

The specificity and selectivity of the proposed methods was evaluated by determining the concentration of esomeprazole magnesium in the presence of

excipients such as glyceryl monostearates, hydroxyl propyl cellulose, magnesium stearate, sugar spheres, talc and triethyl citrate commonly found in tablet formulations of the cited drug. The content of the drug was also determined in the presence of varying amounts of oxidants such as potassium persulfate, ammonium molybdate, sodium metavanadate, and chloramine T.

Linearity

The linearity of the proposed methods was evaluated at the following concentration levels: 2, 4, 8, 16, 20, 24, 36, 40, 44 and 48 μg ml^{-1} for Method A and 10, 20, 30, 40, 50, 70, 90 and 100 μg ml^{-1} for Method B. Each concentration level was repeated five times.

Accuracy and precision

The accuracy and precision of the proposed methods were ascertained at three concentration levels: 8, 24 and 48 μg ml^{-1} for method A and 10, 50 and 100 μg $ml^{-1}$for method B. Five sample solutions of each concentration were analyzed within one day (within day or intra day precision) and in five consecutive days (between day or inter day precision).

Recovery experiments

The recovery studies were carried out by standard addition technique. In this technique, 4.0 ml (or 3.0 ml) of 0.2 mg ml^{-1} solution (or 0.5 mg ml^{-1}) esomeprazole magnesium was mixed separately with 4.0 and 8.0 ml (or 3.0 and 6.0 ml) of 0.2 mg ml^{-1} (or 0.5 mg ml^{-1}) reference esomeprazole magnesium in 50 ml standard volumetric flask and diluted to the volume with methanol (or acetone). The total amount of esomeprazole magnesium was determined by the proposed procedures.

Limits of detection (LOD) and quantitation (LOQ)

The limits of detection and quantitation [5] were calculated using the relations: LOD = 3.3 × S_0 / b and LOQ = 10 × S_0 / b where S_0 and b are the standard deviation and the slope of the calibration line, respectively.

Ruggedness and robustness

The ruggedness of proposed methods was ascertained using two different spectrophotometers (spectronic $20D^+$ and shimadzu uv-visible spectrophotometers). The robustness of methods A and B was evaluated by observing the influence of small variations of experimental variables i.e. concentration of reagents, reaction temperature and heating time.

Evaluation of statistical equivalence testing

The statistical equivalence testing has been evaluated by means of point and interval hypothesis tests [6]. Interval hypothesis test, the test method is compared with a reference method and considered to be acceptable if the mean recovery is within ± 2.0 % of that of the reference method [7]. This can be written as: $0.98 < \mu_2 / \mu_1 < 1.02$ where μ_1 and μ_2 are the population means of the reference and test methods, respectively. This can be generalized in a simpler form as: $\theta_L < \mu_2 / \mu_1 < \theta_U$ where θ_L and θ_U are the lower and upper acceptance limits, respectively. The limits of this confidence interval are obtained from the following quadratic equation:

$$\theta^2\left(\overline{x_1^2} - S_p^2 t_{tab}{}^2 / n_1\right) + \theta(-2\overline{x_1}\,\overline{x_2}) + \left(\overline{x_2^2} - S_p^2 t_{tab}{}^2 / n_2\right) = 0$$

RESULTS AND DISCUSSION

The methanolic solutions of esomeprazole magnesium and 5-sulphosalicylic acid were found to absorb maximally at 301.5 and 215 nm, respectively. Esomeprazole magnesium was allowed to react with 5-sulphosalicylic acid resulting

in the formation of yellow coloured product peaking at 365 nm (**Fig. 4.1**). The literature survey revealed that magnesium is capable of forming coloured mixed ligand complexes with 4,5-dibromophenyl fluorone and cetyltrimethylammonium bromide [8], o-[(1,8-hidroxy-3,6-disulpho-2-naphthyl) azo] benzene arsonic acid and [ethylene bis(oxyethylenenitrilo)] tetracetic acid [9], nitrilotriacetate and 5-nitrosalicylic acid [10]. In the similar manner, magnesium bound esomeprazole as a drug reacts with 5-sulphosalicylic acid in methanol to form a yellow coloured complex which absorbed maximally at 365 nm.

The composition of the complex (drug-Mg (II)-5-sulphosalicylic acid) was determined by mole ratio method [11]. The results are shown in **Fig. 4.2**, which indicated the esomeprazole magnesium-5-sulphosalicylic acid ratio of 1:4. On the basis of our experimental findings and literature background, the reaction sequence is shown in **Scheme 4.1**.

Esomeprazole magnesium was found to react with N-bromosuccinimide in acetone, which yielded a product showing a new characteristic band peaking at 380 nm (**Fig. 4.3**). It was reported that N-bromosuccinimide is a brominating agent useful in the α- bromination of sulphoxide derivative [12]. Esomeprazole magnesium is a base consisting of α-carbon as a prochiral center adjacent to the sulphoxide undergoes reaction with N-bromosuccinimide to form yellow α-bromosulphoxide derivative of the drug. The reaction product absorbed maximally at 380 nm. The mole ratio method was used to evaluate the composition of product and the results are shown in **Fig. 4.4**. As can be seen from the figure that the combining molar ratio between esomeprazole magnesium and N-bromosuccinimide is 1:2. On this basis, the reaction sequence is given in **Scheme 4.2**.

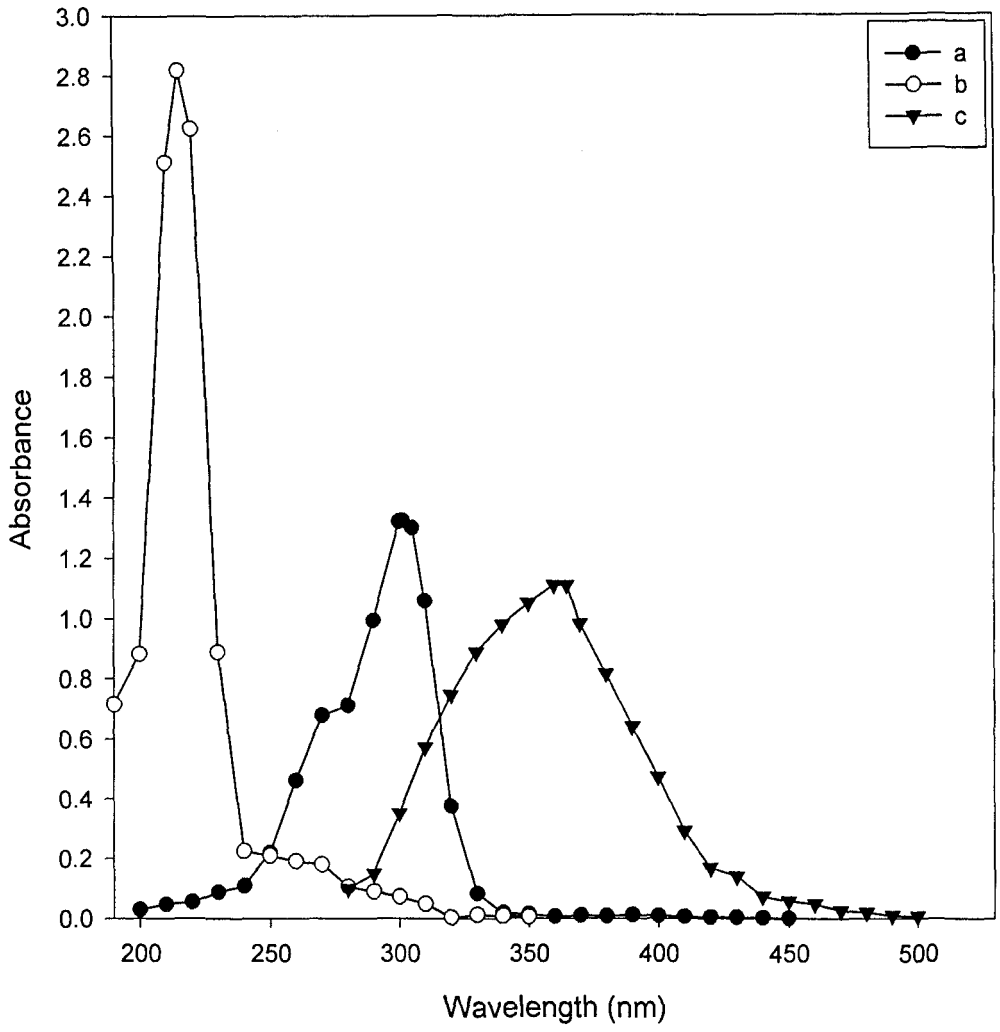


Fig. 4.1. Absorption spectra of (a) 5.213 $\times 10^{-5}$ M (40.0 μg ml^{-1}) esomeprazole magnesium (b) 1.968$\times 10^{-3}$ M 5-sulphosalicylic acid, and (c) 5.213 $\times 10^{-5}$ M (40.0 μg ml^{-1}) esomeprazole magnesium with 3.933 $\times 10^{-4}$ M 5-sulphosalicylic acid in methanol.

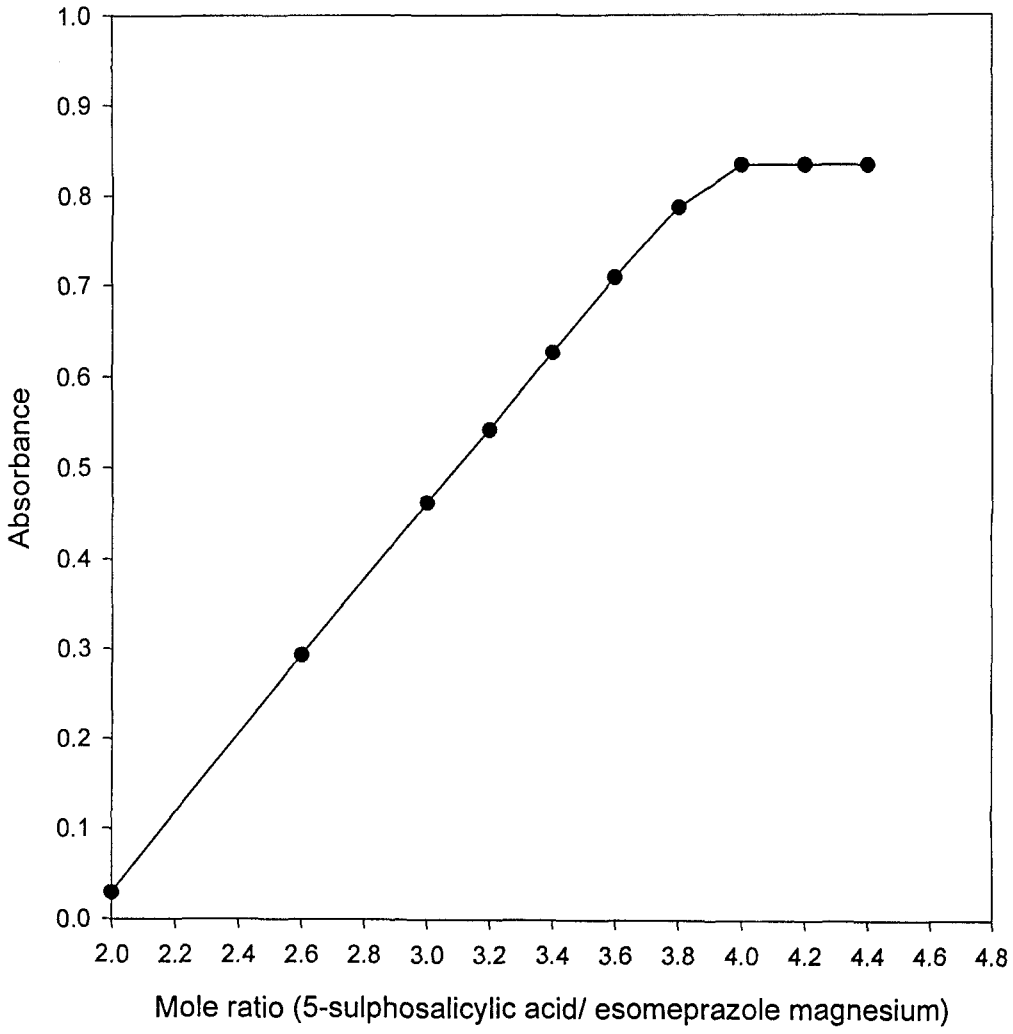
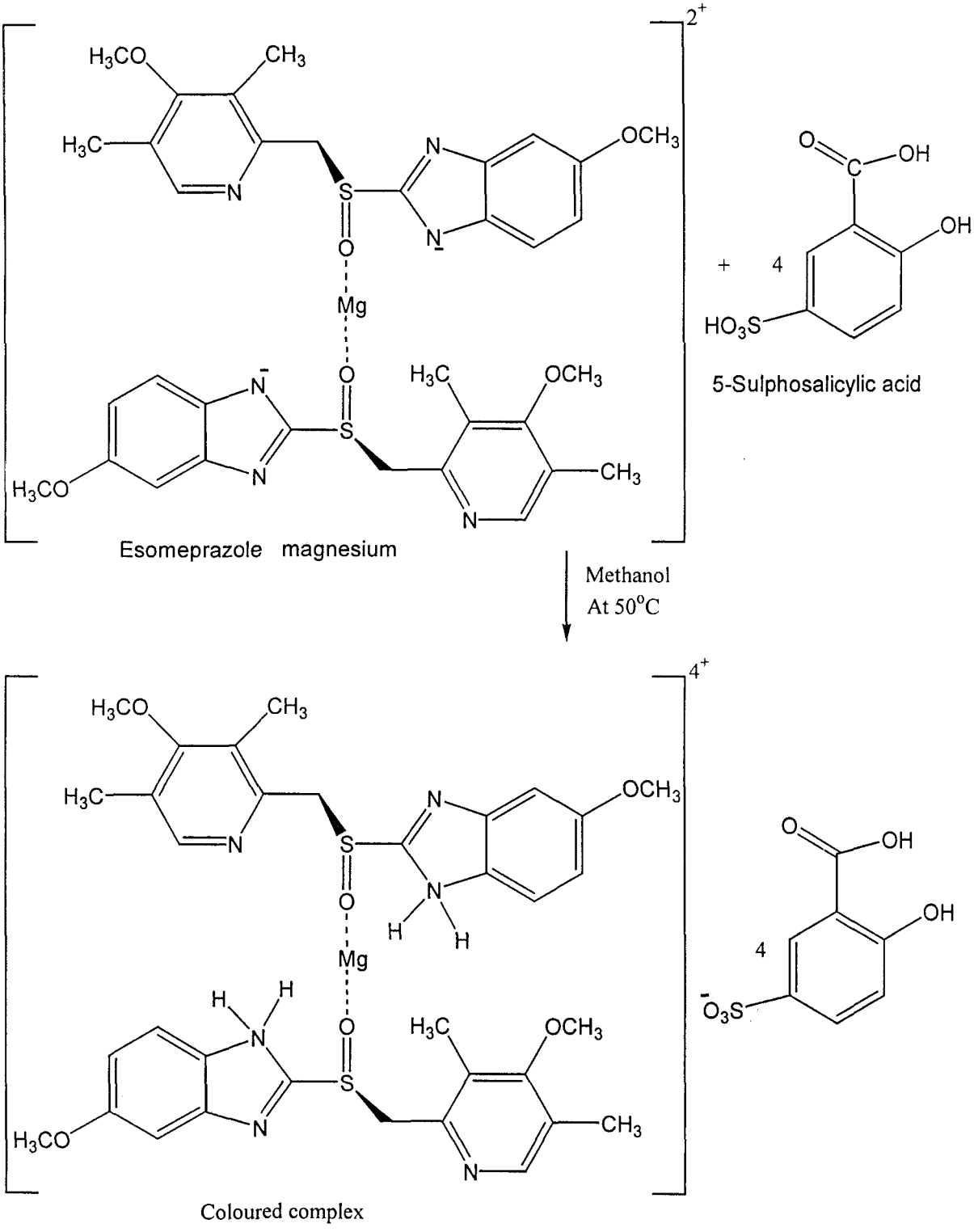


Fig. 4.2. Mole ratio plot for stoichiometric ratio between esomeprazole magnesium and 5-sulphosalicylic acid for method A.


2+
H3CO
CH3
H3C
N
S
O
N
OCH3
Mg
O
H3C
OCH3
N
S
N
H3CO
CH3
N
Esomeprazole magnesium
O
C
OH
OH
+ 4
HO3S
5-Sulphosalicylic acid
Methanol
At 50°C
4+
H3CO
CH3
H3C
N
S
O
N
OCH3
H
H
Mg
H
H
N
O
H3C
OCH3
S
N
H3CO
CH3
N
Coloured complex
O
OH
OH
4
O3S

Scheme 4.1

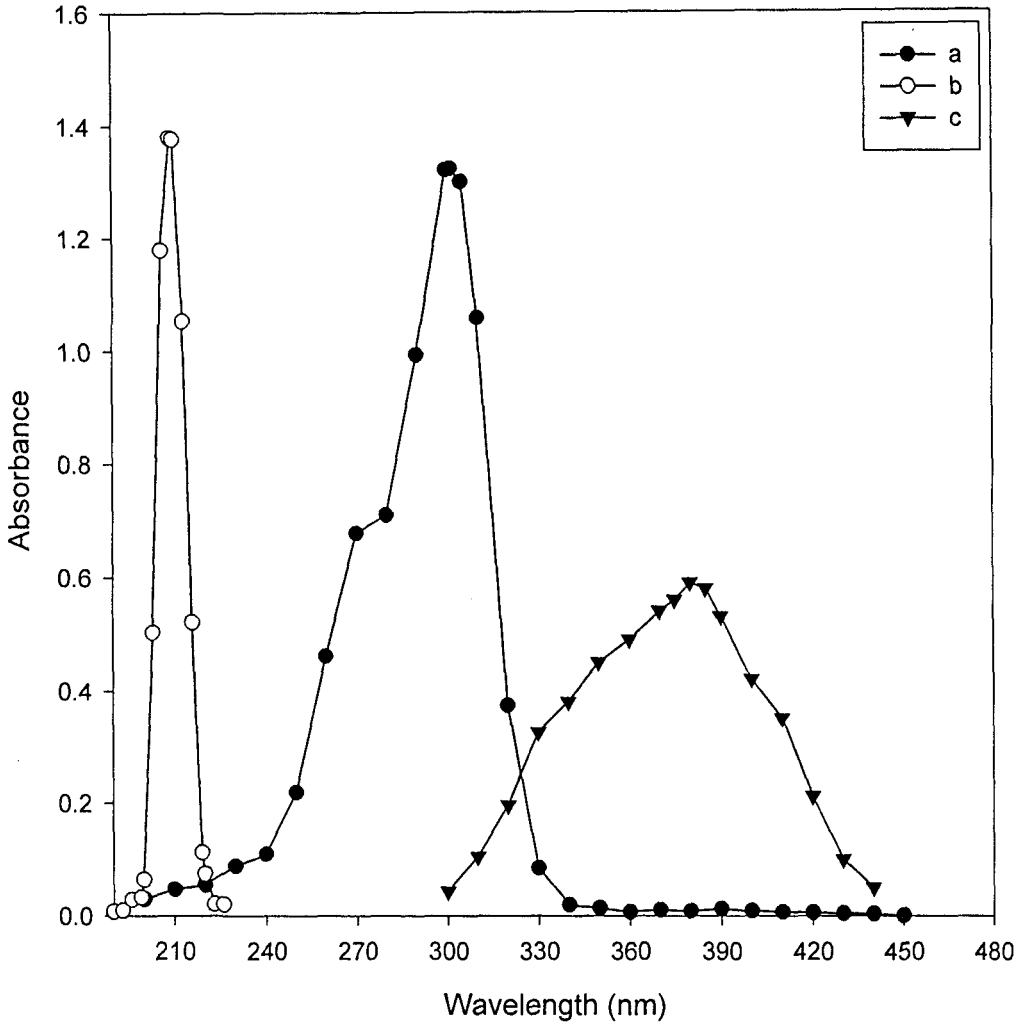


Fig. 4.3. Absorption spectra of (a) 5.213 × 10^{-5} M (40.0 μg ml^{-1}) esomeprazole magnesium in chloroform (b) 2.247 × 10^{-2} M N-bromosuccinimide in acetone and (c) 1.303 × 10^{-4} M (100.0 μg ml^{-1}) esomeprazole magnesium with 9.90 × 10^{-3} M N-bromosuccinimide in acetone.

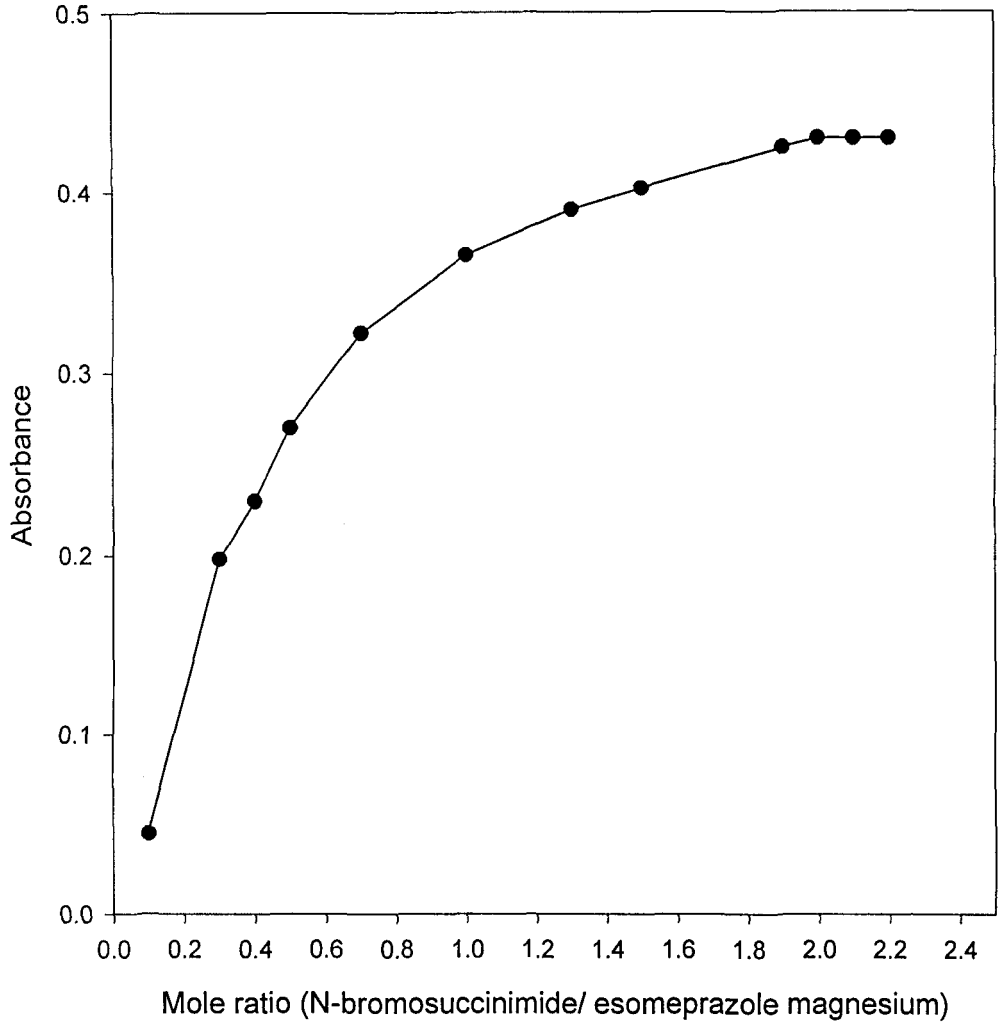
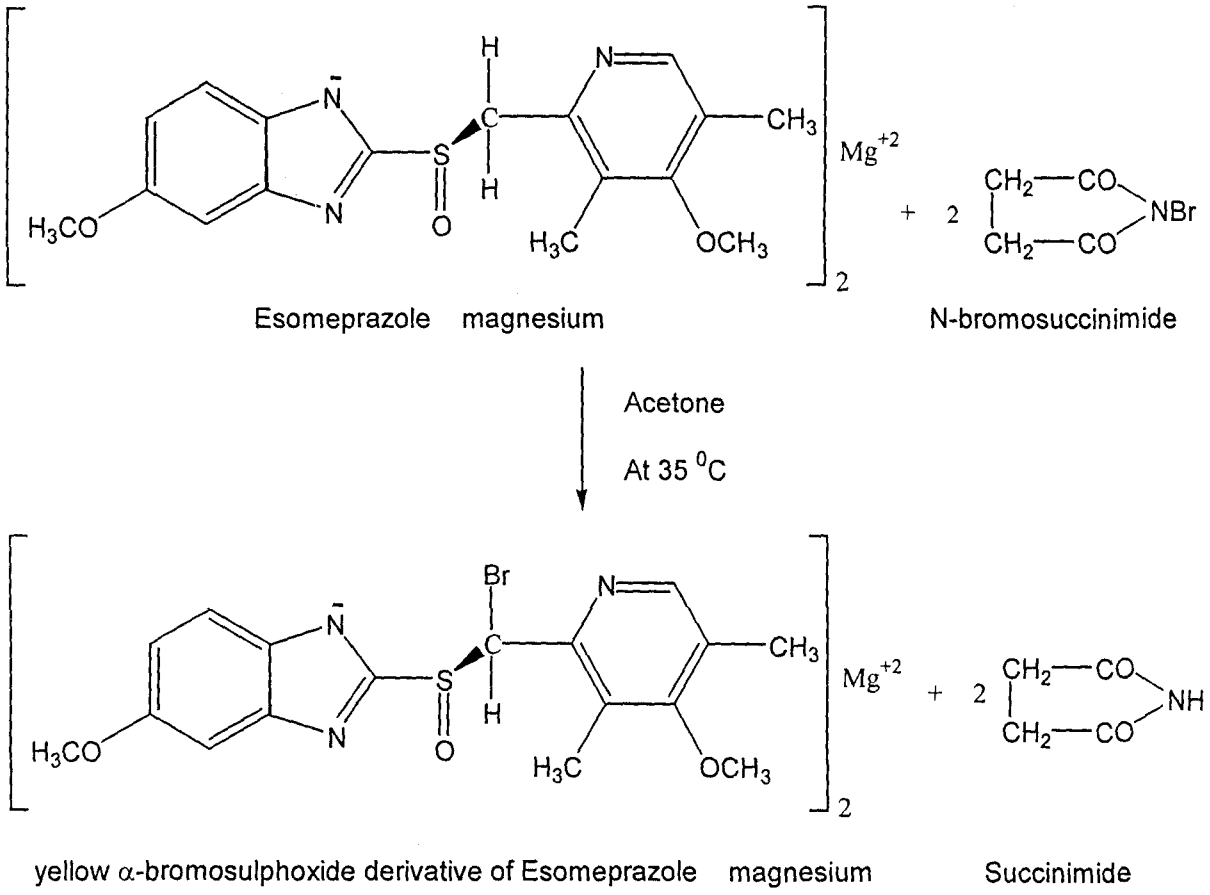


Fig. 4.4. Mole ratio plot for stoichiometric ratio between esomeprazole magnesium and N-bromosuccinimide for method B.


Esomeprazole magnesium
N-bromosuccinimide
Acetone
At 35 ^{0}C
yellow α-bromosulphoxide derivative of Esomeprazole magnesium
Succinimide

Scheme 4.2

TLC of esomeprazole magnesium and its reaction products

The esomeprazole magnesium and its reaction products were analyzed by thin layer chromatography. The sample solution of reference drug and the products of Method A and B were applied on TLC plates coated with silica gel G (Merck, India) as stationary phase. The chromatogram was developed in dichloromethane-methanol-2.67 M ammonia (5: 0.3: 2 v / v / v) solvent system. The plates were air-dried and spots were located on placing the plates in iodine chamber. The spots of pure drug, reaction products of method A and B were detected with R_f values of 0.77, 0.54 and 0.67, respectively.

Optimization of variables

The optimum conditions for methods A and B have been evaluated by studying the reaction as a function of temperature, heating time, concentration of reagents, nature of the solvent and the stability of the coloured species.

Method A

Effect of temperature

The effect of temperature on the absorbance of the yellow coloured product was studied in the range of 40-55°C. It was observed that the rate of reaction increases with increase in temperature. At temperature > 50°C, the reaction product may decompose and affect the reproducibility of the proposed method. Therefore to minimize the time of analysis and for the sake of good results, a temperature of 50°C was selected for the determination process.

Effect of time

To study the effect of time on the absorbance of the coloured product, 1 ml of esomeprazole magnesium (0.2mg ml^{-1}) was mixed with 1.2 ml of 5-sulphosalicylic acid (0.5mg ml^{-1}) and kept for different time interval in a water bath maintained at

50 ± 1°C. The contents of mixture were diluted to 5 ml with methanol after cooling at room temperature (25 ±1°C). It is apparent from the **Figure 4.5** that the maximum absorbance was obtained at 4 min and remained constant up to 6 min. Therefore, 5 min of heating at 50 °C was chosen as an optimum value.

Effect of concentration of 5-sulphosalicylic acid

The effect of 5-sulphosalicylic acid concentration on the absorbance of the coloured product was investigated in the range of 3.93×10^{-5}- 5.51×10^{-4} M keeping a constant amount of drug (200 µg). It was found that the absorbance of the coloured product increased with increasing concentration of 5-sulphosalicylic acid and became constant in the concentration range of 3.93×10^{-4} - 5.51×10^{-4} M (**Fig. 4.6**). Thus 4.72×10^{-4} M 5-sulphosalicylic acid was adopted as most suitable concentration for maximum absorbance.

Method B

Effect of temperature

The effect of temperature on the absorbance of the coloured product was investigated over the range 25 - 40°C. It was found that the suitable temperature of colour development was 35°C. Therefore, for the sake of good recovery results, the optimum temperature of 35°C was selected for the determination procedure.

Effect of time

To study the effect of time for the maximum absorbance, 500 µg esomeprazole magnesium was mixed with 2.2 ml of 2.25×10^{-2} M N-bromosuccinimide and allowed to stand at 35 ±1°C. The absorbance was measured at 380 nm as a function of time. It was apparent from **Fig 4.7** that the absorbance

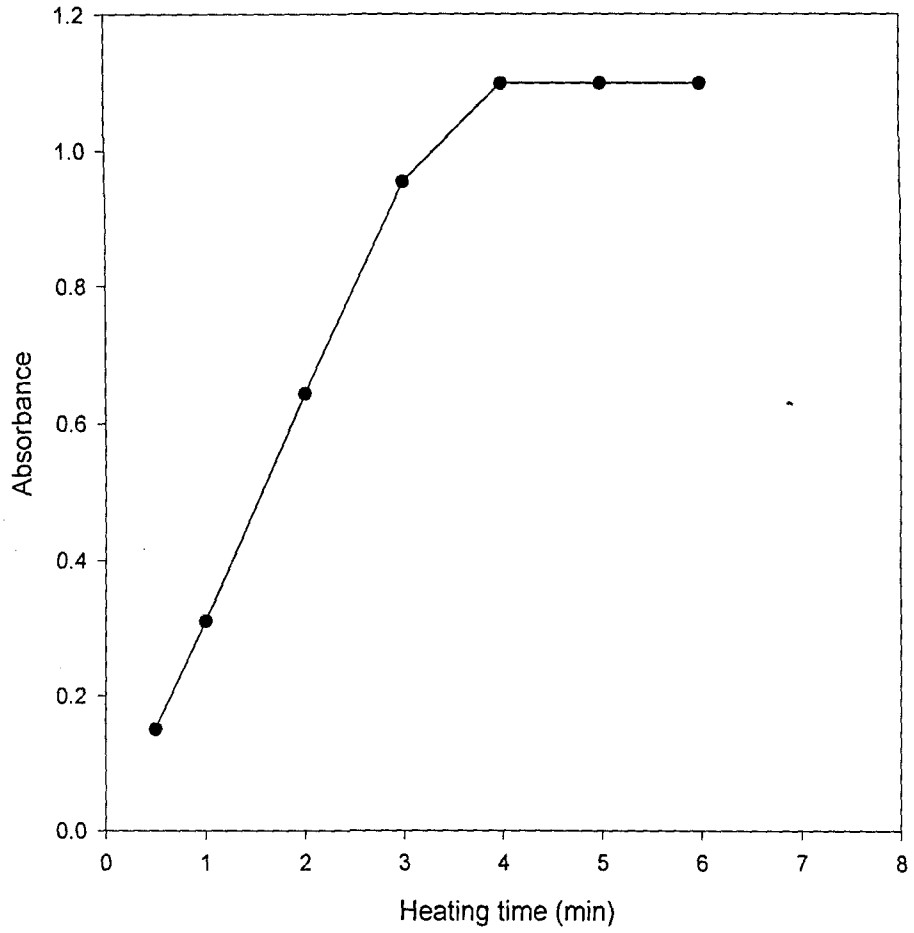


Fig. 4.5. Effect of time on the absorbance for Method A.

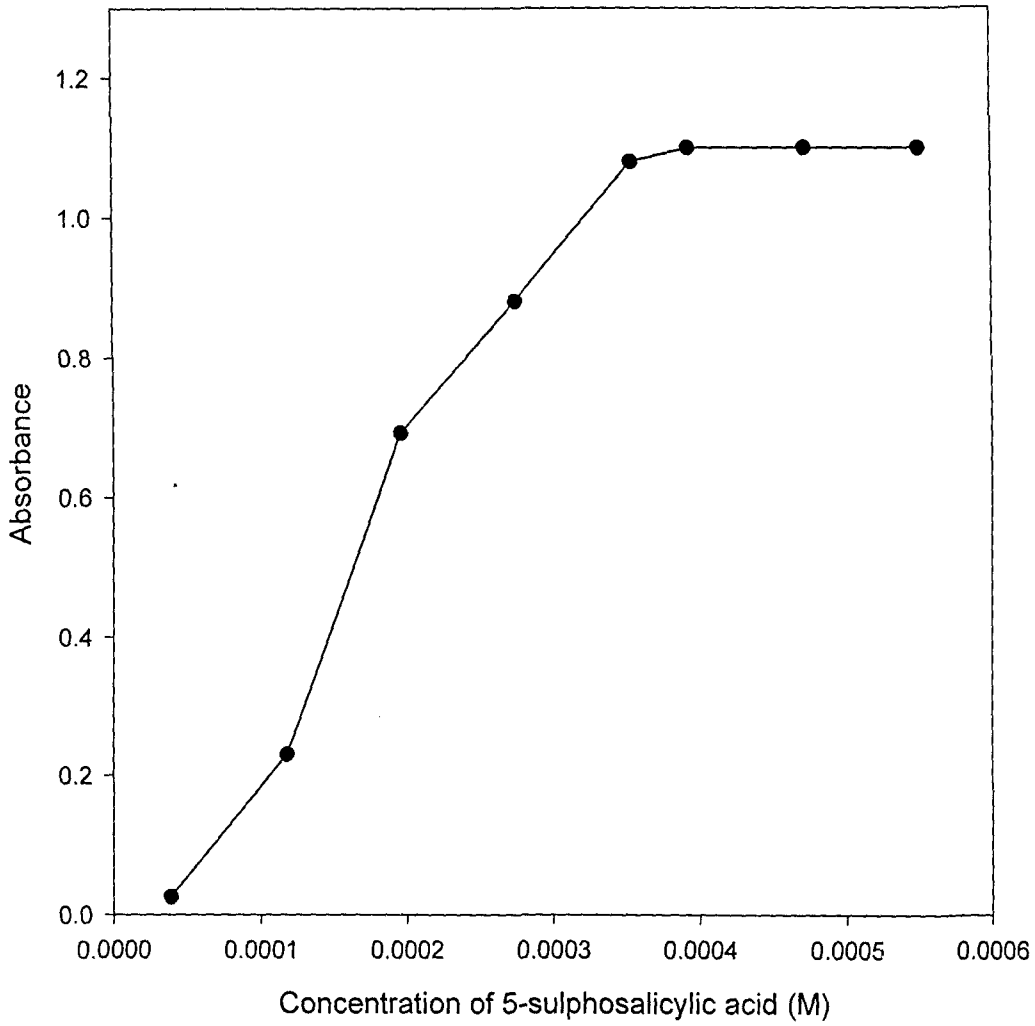


Fig. 4.6. Effect of the concentration of 5-sulphosalicylic acid on the absorbance for method A keeping [Esomeprazole magnesium] = 40.0 μg ml^{-1}.

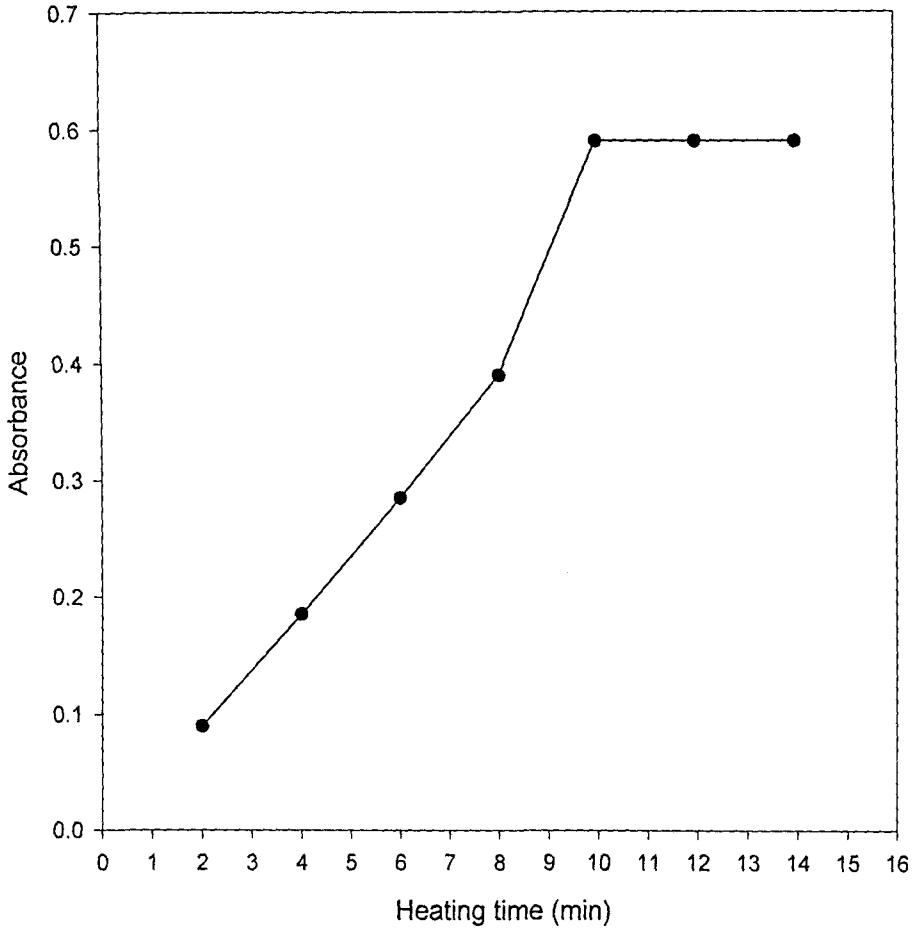


Fig. 4.7. Effect of time on the absorbance for Method B.

remained constant between 10 and 14 min at 35 ± 1°C. Therefore, the reaction time of 12 min was chosen for the estimation of esomeprazole magnesium.

Effect of the concentration of N-bromosuccinimide

The effect of the concentration of N-bromosuccinimide on the absorbance of the coloured product was critically examined in the range of 4.50×10^{-4} - 1.08×10^{-2} M. It was found that increasing the concentration of N-bromosuccinimide would increase the absorbance of the reaction up to 9.00×10^{-3} M; above this concentration absorbance remained constant (**Fig 4.8**). Therefore, the concentration of 9.90×10^{-3} M N-bromosuccinimide was adopted as an optimum value throughout the determination process.

Solution stability

It is reported that esomeprazole magnesium has acceptable stability under alkaline conditions [13]. The drug is stable in solvents like methanol and chloroform. The test and quality control sample solutions showed no change in the absorption spectra for three days at room temperature (25±1°C). A single spot was resulted in test solution as well as in quality control sample solutions with $R_f = 0.77$, which corresponds to esomeprazole magnesium.

Specificity and selectivity

It was found that the common excipients (glyceryl monostearates, hydroxyl propyl cellulose, magnesium stearate, sugar spheres, talc and triethyl citrate) did not interfere with the proposed procedures. The recovery of drug in presence of oxidants such as potassium persulphate, ammonium molybdate, sodium metavanadate and

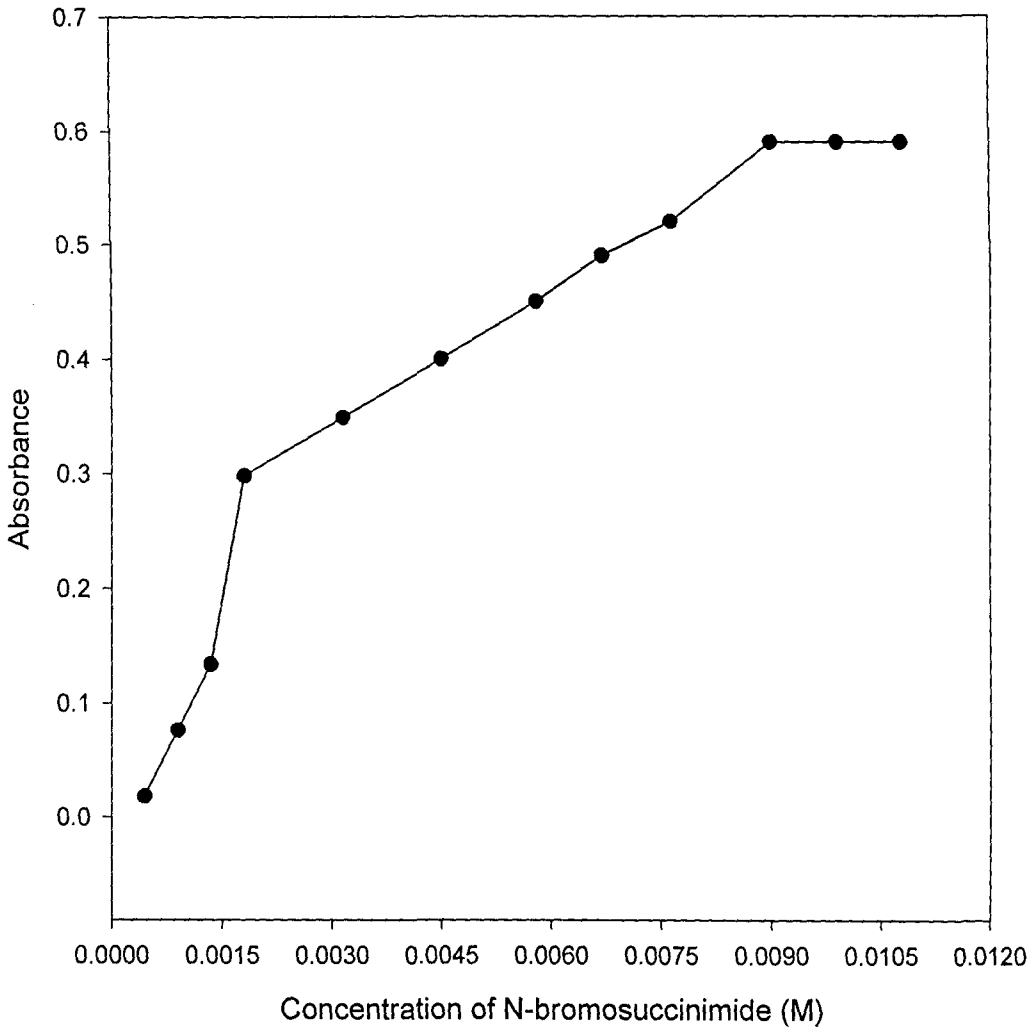


Fig. 4.8. Effect of the concentration of N-bromosuccinimide on the absorbance for method B keeping [esomeprazole magnesium] = 100.0 μg ml^{-1}.

chloramine T was quite satisfactory by both the procedures.

Ruggedness and robustness

The ruggedness of the proposed methods was assessed by analyzing the active esomeprazole magnesium in Nexpro-20 tablets at 20 μg ml^{-1} using two different spectrophotometers. The mean % recovery ± RSD was found to vary over the range 99.98 ± 0.32 - 100.06 ± 0.35.

The robustness of the proposed methods relative to each operational parameter was closely monitored. The operational parameters examined were as follows:

Method A

- heating temperature, 50 ± 1°C
- heating time, 5 min (± 1 min)
- 1.97×10^{-3} M 5-sulphosalicylic acid, 1.2 ml (± 0.2 ml)

Method B

- heating temperature, 35 ± 1°C
- heating time, 12 min (± 2min)
- 2.25×10^{-2} M N-bromosuccinimide, 2.2 ml (± 0.2 ml)

The robustness of the proposed methods (A and B) was judged by analyzing the active drug contents in tablet formulations (Nexpro 20) under delibrate small changes in experimental conditions. A quality control sample solution containing 20 μg ml^{-1} of the drug was analyzed five times using methods A and B. The results showed that the mean % recovery ± RSD was found to be 99.98 ± 0.34 and 100.02 ± 0.35 for methods A and B, respectively.

Analytical performance of the proposed methods

Under the optimized experimental conditions, the absorbance values for methods A and B were found to be proportional to the concentration of esomeprazole magnesium over the ranges 2 - 48 and 10 - 100 μg ml^{-1} with molar absorptivity of 2.113×10^4 and 4.565×10^4 l mol^{-1} cm^{-1}, respectively. The regression equation, correlation coefficient, limits of detection and quantitation, confidence interval of intercept and slope at 95% confidence level and variance (S_o^2) of calibration line were evaluated and summarized in **Table 4.1**. In both the methods, the value of correlation coefficient was 0.9999, which indicated the good linearity of calibration lines. The low values of variance of calibration lines (8.47×10^{-6} and 6.62×10^{-7} for methods A and B, respectively) pointed towards good reproducibility of the procedures. It is drawn out from the relation, $t = a/S_a$ [14], that the values of the intercept of the regression lines are close to zero. The student's t-test has confirmed that the experimental intercepts, a, for methods A and B are not significantly different from the theoretical value of zero because the t-values calculated from the above relation were found to be 0.152 and 1.074 for methods A and B, respectively which did not exceed the tabulated t-values, $t = 2.306$, $\nu = 8$ for method A and $t = 2.447$, $\nu = 6$ for method B at 95% confidence level. Therefore, it is concluded that the proposed procedures are completely free from constant errors.

The accuracy and precision of the proposed methods A and B were evaluated at three concentration levels: 8, 24, 48 μg ml^{-1} and 10, 50 and 100 μg ml^{-1}, respectively. The % relative error and relative standard deviation are summarized in **Table 4.2**. As can be seen from **Table 4.2** that the % relative error and relative standard deviation were ≤ 0.4 and 0.62 %, respectively. The results of the recovery experiments are summarized in **Table 4.3**. It is evident from the table that %

Table 4.1
Optical and validation data for the determination of esomeprazole magnesium

Parameters	Method A	Method B
Wavelength (nm)	365	380
Beer's law limit (μg ml^{-1})	2 - 48	10 - 100
Molar absorptivity (l $mol^{-1}cm^{-1}$)	2.113×10^4	4.565×10^4
Linear regression equation	$A = 2.527 \times 10^{-4} + 2.75 \times 10^{-2} C$	$A = 6.027 \times 10^{-4} + 5.90 \times 10^{-3} C$
$\pm tS_a$	4.608×10^{-3}	1.558×10^{-3}
$\pm tS_b$	1.584×10^{-4}	2.609×10^{-5}
Correlation coefficient (r)	0.9999	0.9999
Variance (S_0^2) of calibration line	8.47×10^{-6}	6.62×10^{-7}
Detection limit (μg ml^{-1})	0.35	0.46
Quantitation limit (μg ml^{-1})	1.06	1.38

$\pm t\ S_a$ and $\pm t\ S_b$ are confidence limits for intercept and slope, respectively

Table 4.2
Test of accuracy and precision of the proposed methods

Proposed methods	Concentration ($\mu g\ ml^{-1}$)		Error (%)	RSD[a] (%)	SAE[b]	C.L.[c]
	Taken	Found ± SD[a]				
Method A						
Intra day assay	8	8.01 ± 0.04	0.12	0.494	0.018	0.050
	24	24.04 ± 0.04	0.16	0.173	0.019	0.052
	48	48.03 ± 0.08	0.06	0.166	0.036	0.099
Inter day assay	8	8.02 ± 0.04	0.25	0.550	0.019	0.055
	24	24.01 ± 0.05	0.04	0.186	0.020	0.056
	48	48.04 ± 0.08	0.08	0.162	0.035	0.096
Method B						
Intra day assay	10	10.03 ± 0.05	0.30	0.512	0.064	0.064
	50	49.98 ± 0.08	0.04	0.149	0.034	0.093
	100	100.01 ± 0.07	0.01	0.069	0.031	0.087
Inter day assay	10	10.04 ± 0.06	0.40	0.621	0.028	0.077
	50	50.01 ± 0.11	0.02	0.220	0.049	0.137
	100	100.04 ± 0.07	0.04	0.074	0.033	0.092

[a]Mean for five independent determinations.
[b]SAE, standard analytical error.
[c]C.L., confidence limit at 95% confidence level and four degrees of freedom (t = 2.776).

Table 4.3
Determination of esomeprazole in pharmaceutical formulations by standard addition technique

Formulations	Method A Concentration ($\mu g\ ml^{-1}$)						Method B Concentration ($\mu g\ ml^{-1}$)					
	Taken	Added	Found ± SD[a]	Recovery (%)	RSD[a] (%)	C.L.[b]	Taken	Added	Found ± SD[a]	Recovery (%)	RSD[a] (%)	C.L.[b]
Nexpro-20	16	16	32.00 ± 0.05	100.03	0.162	0.065	30	30	59.99 ± 0.06	99.99	0.106	0.079
(Torrent)	16	32	48.04 ± 0.08	100.08	0.158	0.094	30	60	90.00 ± 0.07	100.00	0.077	0.086
Raciper-20	16	16	31.99 ± 0.07	99.99	0.021	0.083	30	30	59.98 ± 0.06	99.98	0.093	0.069
(Ranbaxy)	16	32	48.02 ± 0.08	100.06	0.166	0.098	30	60	89.99 ± 0.07	99.99	0.079	0.088
Esoz-20	16	16	32.01 ± 0.05	100.01	0.164	0.066	30	30	59.99 ± 0.06	99.99	0.099	0.074
(Glenmark)	16	32	48.03 ± 0.08	100.07	0.159	0.095	30	60	90.00 ± 0.07	100.00	0.078	0.087

[a]Mean for five independent determinations.
[b]C.L., confidence limit at 95% confidence level and four degrees of freedom (t = 2.776).

recovery and relative standard deviation were in the range of 99.99 - 100.07 % and 0.02 - 0.17% for method A and 99.98 - 100.01 % and 0.08 - 0.11% for method B, respectively. These results indicated that there is no interference from the common excipients presents in tablet formulations. The proposed methods and reference method were applied to the determination of esomeprazole magnesium in tablet formulations. The results obtained by the proposed methods were compared statistically with those obtained by the reference method (**Table 4.4**). The assay results showed that student's t- and F- values did not exceed the theoretical ones at 95% confidence level. In all cases, the values of lower and upper limits based on recovery experiments were found to be within ± 2%.

CONCLUSION

The drug is a complex of magnesium, which selectively reacts with 5-sulphosalicylic acid in methanol to form coloured complex peaking at 365 nm (method A). The drug is showing no sign of reaction with reagents devoid of sulfonic group such as salicylic acid, benzoic acid and phenol. Method B is selective as the drug is containing carbon as a prochiral center (α-carbon) adjacent to sulphoxide preferentially reacts with N-bromosuccinimide in acetone, hence undergoes α-bromination of drug, which absorbed maximally at 380 nm. The drug gives positive response only to those oxidizing agents, which contain Br in their moiety. Hence, the two methods are selective, but method A is more superior due to the formation of ternary complex and rapid analysis (5 min) with low detection limit (0.35 μg ml^{-1}) compared to method B, requiring more analysis time (12 min) with high limit of detection (0.46 μg ml^{-1}). Therefore methods A and B can be used for the routine quality control analysis of esomeprazole magnesium in industries, research laboratories and hospitals.

Table 4.4
Analysis of esomeprazole magnesium of the proposed methods using point and interval hypothesis tests at 95% confidence level

Formulations	Method A		Method B		Reference method	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Nexpro-20 (Torrent)	99.98 θ_L=0.988 t =0.09	0.33 θ_U=1.011 F= 1.01	99.98 θ_L=0.987 t =0.30	0.34 θ_U=1.012 F= 1.08	100.02	0.35
Raciper-20 (Ranbaxy)	100.06 θ_L=0.989 t =0.18	0.33 θ_U=1.012 F= 1.02	100.01 θ_L=0.992 t =0.59	0.35 θ_U=1.009 F= 1.05	99.98	0.33
Esoz-20 (Glenmark)	99. 99 θ_L=0.987 t =0.04	0.35 θ_U=1.012 F= 1.17	100. 01 θ_L=0.988 t =0.05	0.32 θ_U=1.012 F= 1.09	100.01	0.36

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CHAPTER-5

OPTIMIZATION AND VALIDATION OF SPECTROPHOTOMETRIC METHODS FOR THE QUANTITATION OF RABEPRAZOLE SODIUM IN COMMERCIAL DOSAGE FORMS

INTRODUCTION

Rabeprazole sodium is chemically known as 2-({[4-(3-methoxy propoxy)-3-methyl-2-pyridyl] methyl} sulfinyl)-1H-benzimidazole sodium ($C_{18}H_{20}N_3NaO_3S$ = 381.4). Rabeprazole sodium represents the newest class of antisecretory agents that are well known for their proton pump (H^+ / K^+-ATPase) inhibitor activity, most profoundly diminishing gastric acid secretion and thus, lowering the luminal concentration of hydrogen ions. It has recently been demonstrated that rabeprazole sodium is the only proton pump inhibitor among tested (omeprazole, lansoprazole) that augments gastric mucin content [1]. It has proven efficacy in healing, symptom relief and prevention of relapse peptic ulcers and gastro-oesophageal reflux disease. It is an important alternative to H_2 antagonists and an additional treatment option to other proton pump inhibitors in the management of acid related disorders.

The drug is officially listed in Martindale The Extra Pharmacopoeia [2]. The assay of drug in bulk and formulations is not cited in the United States Pharmacopoeia or British Pharmacopoeia. In view of the great importance and wide use of rabeprazole sodium, different analytical methods have been reported for its determination which include high performance liquid chromatography [3-5], liquid chromatography coupled with tandem mass spectrometry [6], capillary electrophoresis [7], and UV-spectrophotometry [8]. These reported methods are sensitive but expensive and require laboratories clean up procedure prior to analysis of drug. Moreover, UV spectrophotometry requires the elimination of matrix effects, since many compounds present in tablet formulations also absorb in UV region. These elimination efforts make the method more complicated and laborious. Spectrophotometry in visible region is attractive because of speed, and simplicity. Extractive spectrophotometric methods have been utilized for the quantitation of

rabeprazole sodium in pharmaceutical formulations based on chloroform extractable ion pair complexes of the drug with bromothymol blue, bromocresol green, bromocresol purple, amido black and alzarin Red S in acidic medium at 424, 430, 422, 636 and 437 nm, respectively [9].

This chapter describes two visible spectrophotometric methods for the determination of rabeprazole sodium in commercial dosage forms. Method A is based on the reaction of rabeprazole sodium with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) in the presence of ammonium cerium (IV) nitrate in acetic acid medium to form coloured species which absorbs maximally at 470 nm. Method B utilizes the reaction of rabeprazole sodium with 1-chloro-2,4-dinitrobenzene (CDNB) in dimethyl sulphoxide (DMSO) to form Meisenheimer complex peaking at 420 nm. The reaction conditions are optimized and validated as per ICH guidelines [10].

EXPERIMENTAL

Apparatus

Shimadzu (UV-1240, Shimadzu Corporation, Kyoto, Japan) and Milton Roy Company ($20D^+$, USA) spectrophotometers were used for absorbance measurements.

Reagents and Materials

All chemicals used were of analytical reagent grade.

- MBTH (s.d. fine-chem Ltd., Mumbai, India) solution (1.7×10^{-3} M) was freshly prepared in distilled water.
- Ammonium cerium (IV) nitrate (Fluka Chemie AG) solution (2.0×10^{-2} M) was prepared in 3.5×10^{-2} M acetic acid (Merck, India).

- CDNB (Fluka Chemie AG) solution (5.59×10^{-2} M) was prepared in DMSO (Merck, India).

Rabeprazole sodium reference standard was supplied by Hetero Drug Ltd., Hyderabad, India (Batch No.: RSO250305). Tablet formulations of rabeprazole sodium such as Rabicip-20 (Cipla, Mumbai, India), Rablet-20 (Lupin, Mumbai, India), Rapeed-20 (Alkem, Mumbai, India) were purchased from local drug stores.

Test solutions

- Rabeprazole sodium (1 mg ml^{-1}) solution was prepared in distilled water.
- Rabeprazole sodium (0.75 mg ml^{-1}) solution was prepared in DMSO.

Proposed Procedures for the quantitation of rabeprazole sodium

Method A

Aliquots (0.14 - 1.4 ml) of standard rabeprazole sodium (1 mg ml^{-1}) solution corresponding to 14 - 140 μg ml^{-1} were pipetted into a series of 10 ml volumetric flasks. To each flask, 1.7 ml of 2.0×10^{-2} M ammonium cerium (IV) nitrate and 2.0 ml of 1.71×10^{-3} M MBTH were added and diluted to volume with distilled water. The contents of the flask were mixed well and kept for 10 min at room temperature (25 ± 1°C) to complete the reaction. The absorbance of each solution was measured at 470 nm against the reagent blank prepared simultaneously except drug within the stability time period of 6 h. The amount of the drug was calculated either from the calibration graph or the corresponding regression equation.

Method B

Into a series of boiling test tubes, different volumes (0.05 - 1.1 ml) of standard rabeprazole sodium (0.75 mg ml^{-1}) solution corresponding to 7.5 - 165 μg ml^{-1} were pipetted. To each test tube, 2.5 ml of 5.92×10^{-3} M CDNB was added, mixed well and heated on water bath for 10 min at 45 ± 1°C. After cooling at room temperature,

the contents of the test tube were transferred to a 5 ml volumetric flask and the volume was completed with DMSO. The absorbance was measured at 420 nm against the reagent blank treated similarly within the stability period of 24 h.

Procedure for the quantitation of rabeprazole sodium in tablet formulations

Five commercially available tablets of 20 mg strength of rabeprazole sodium were taken in distilled water and DMSO separately and kept for 10 min for complete dispersion of the drug. The distilled water and DMSO extracts were filtered through Whatmann No. 42 filter paper (Whatmann International Limited, Kent, UK) in 100 ml volumetric flasks individually. The left residues were washed well with 5 × 10 ml portions of distilled water or DMSO, as the case may be, for complete recovery of the drug and diluted to volume with the corresponding solvent. The amount of drug in commercial tablets was assayed following the proposed procedures.

Procedure for reference method

Aliquots (0.1 - 2.0 ml) of standard rabeprazole sodium (0.5 mg ml^{-1}) corresponding to 10 - 100 μg ml^{-1} were pipetted into a series of 10 ml volumetric flasks and diluting to volume with distilled water. The absorbance was recorded against the solvent blank at 290 nm. The amount of the drug in a given sample can be estimated either from the calibration graph or the corresponding regression equation.

Method validation

Solution stability

The stability of reference rabeprazole sodium and quality control sample solutions at room temperature was evaluated with the help of UV-visible spectra and thin layer chromatography using TLC plates coated with silica gel G (Merck, Mumbai, India) and acetone-toluene-methanol (v/v/v/: 9:9:1.5) as mobile phase.

Specificity and selectivity

The specificity and selectivity of the proposed methods was ascertained by analyzing rabeprazole sodium in presence of excipients such as mannitol, magnesium oxide, low substituted hydroxylpropyl cellulose, magnesium stearate, hypromellose pthalate diacetylated monoglycerides, talc, titanium dioxide, yellow iron oxide and carnauba wax.

Ruggedness and robustness

For the evaluation of ruggedness of methods A and B, the contents of rabeprazole sodium (80 μg ml^{-1}) was analyzed following the proposed procedures (A and B) using Spectronic $20D^{+}$ and Shimadzu UV 1240 spectrophotometers. The two results were compared in terms of % recovery ± RSD.

In the similar manner, proposed methods robustness was evaluated by analyzing rabeprazole sodium (80 μg ml^{-1}) under the influence of small variations of experimental variables. The exactness of each operational parameter was checked by varying one experimental parameter at a time keeping the other parameters constant and the % recovery ± RSD of drug was calculated.

Linearity

For evaluation of linearity, the contents of rabeprazole sodium was determined at nine concentration levels: 14, 15, 20, 30, 40, 60, 100, 120 and 140 μg ml^{-1} for method A and 7.5, 10.5, 12, 15, 75, 90, 125, 150 and 165 μg ml^{-1} for method B. Each concentration was independently analyzed for five times.

Limits of detection and quantitation

The limits of detection and quantitation were evaluated from the following equations:

$$LOD = 3.3 \times \frac{S_0}{b}$$

$$LOQ = 10 \times \frac{S_0}{b}$$

Accuracy and precision

Three concentrations levels of reference rabeprazole sodium solution within the linearity range of methods A and B were selected: 14, 60 and 140 μg ml^{-1} for method A and 10.5, 65 and 165 μg ml^{-1} for method B. Five independent analyses at each concentration level was performed within one day (intra day precision). This analysis was repeated for five consecutive days too (inter day precision).

Equivalence testing

For pharmaceutical analysis, a bias of ± 2.0 % is acceptable [11] and can be calculated statistically [12] using the following quadratic equation:

$$\theta^2\left(\overline{x_1^2} - S_p^2 t_{tab}{}^2 / n_1\right) + \theta(-2\overline{x_1 x_2}) + \left(\overline{x_2^2} - S_p^2 t_{tab}{}^2 / n_2\right) = 0$$

where $\overline{x_1}$ and $\overline{x_2}$ are mean values based on n_1 and n_2 measurements, respectively. S_p is the pooled standard deviation and t_{tab} is the tabulated one-sided t-value, with $n_1 + n_2 - 2$ degrees of freedom at 95% confidence level.

RESULTS AND DISCUSSION

Method A

The literature citation revealed that MBTH on oxidation with cerium (IV) in acidic medium produces an active electrophilic intermediate [13] which further reacts with iminoheteroaromatic compounds such as indole, carbazole, phenothiazine and benzimidazole resulting in the formation of a coloured azo cationic species [14,15]. Benzimidazole is the iminoheteroaromatic compound, which undergoes electrophilic substitution in the benzene ring. The order of substitution is 7 > 6 > 5 > 4 [16]. Rabeprazole sodium is a water soluble proton

pump inhibitor having benzimidazole as the active group and hence undergoes similar electrophilic substitution at position 7 of the benzene ring with the electrophilic intermediate of MBTH in acetic acid medium resulting in the formation of azo cation species, which absorbs maximally at 470 nm. The blank consisting of MBTH and Ce(IV) in acidic medium absorbed at 350 nm. The absorption spectra are shown in **Fig. 5.1.** The combining ratio was evaluated by Bent and French method [17]. The plot of log absorbance *vs* log [rabeprazole sodium] or [MBTH] or [Ce(IV)] gave values of the slopes of 1, 1 and 0.98, respectively **(Fig. 5.2).** Hence it is concluded that the reaction proceeds in the molar ratio of 1:1:1. The reaction sequence is shown in **Scheme 5.1.**

Method B

Polynitroaromatic and halopolynitroaromatic compounds interact with a variety of Bronsted bases to give brightly coloured species due to the activating effect of a nitro group with nucleophilic displacement of an ortho substituent, especially halogen. Therefore, in general addition-elimination mechanism via an intermediate σ, or Meisenheimer complex is accepted [18]. Halogen may be displaced by nitrogen bases (nucleophiles) such as imidazole, benzimidazole, 1,3,5-trimethyl pyrazole and 3,5-dimethyl pyrazole [19]; and piperidine [20]. It was reported that piperidine is a nitrogen base interacted with 1,3,5-trinitrobenzene in DMSO to form coloured species of 1,3,5 - trinitrophenyl piperidine. In this reaction, 2 moles of nitrogen base were utilized with one mole of 1,3,5-trinitrobenzene. Rabeprazole sodium is a nitrogen base due to the presence of benzimidazole group which reacts with CDNB in DMSO at 45 ± 1°C resulting in the formation of

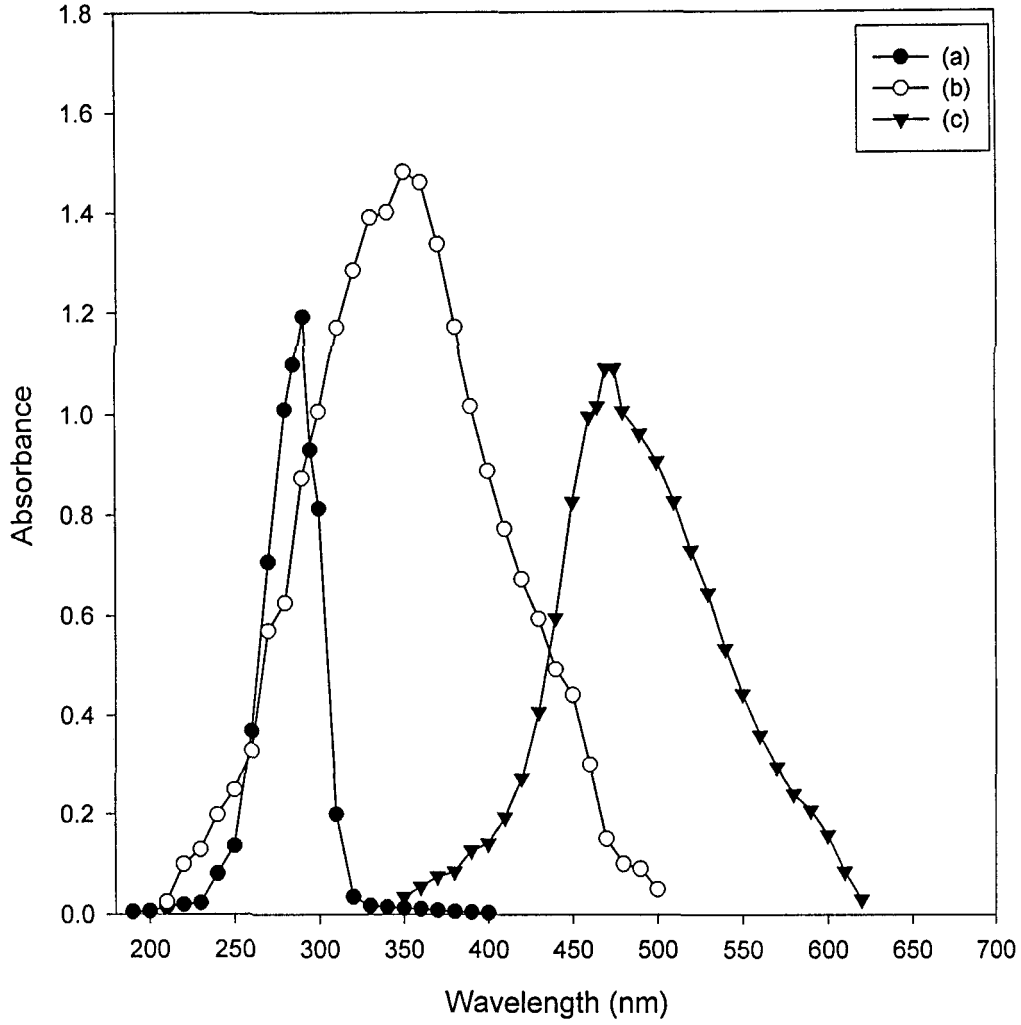


Fig. 5.1. Absorption spectra of (a) rabeprazole sodium (40.0 μg ml^{-1}) in distilled water (b) blank solution: 3.4 $\times10^{-3}$ M ammonium cerium (IV) nitrate and 2.57 $\times10^{-4}$ M MBTH in 5.95 $\times10^{-3}$ M acetic acid (c) sample solution: blank solution + 100.0 μg ml^{-1} rabeprazole sodium.

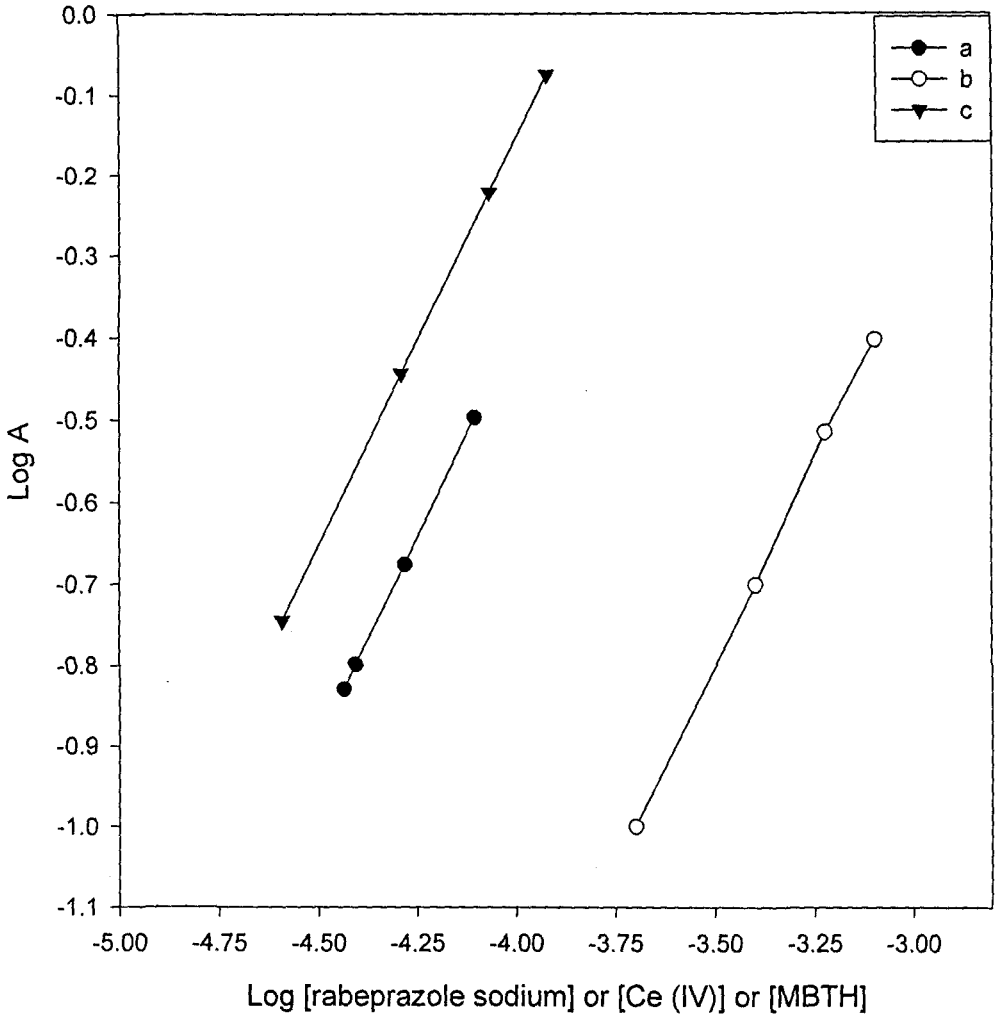
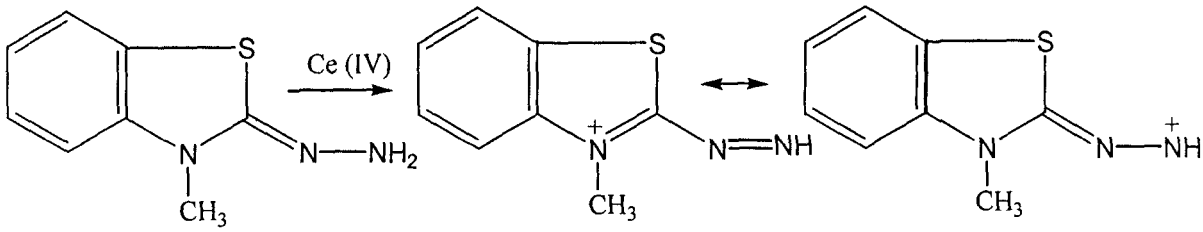


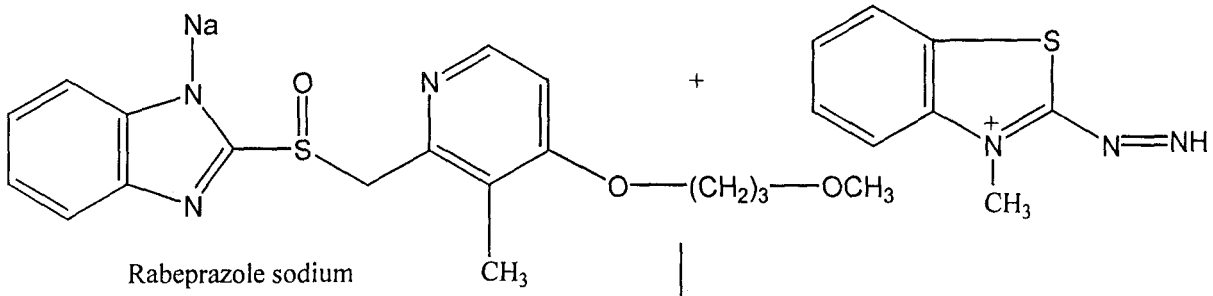
Fig. 5.2. Bent and French stoichiometric plots: (a) rabeprazole sodium, (b) Ce(IV) and (c) MBTH.

Step 1

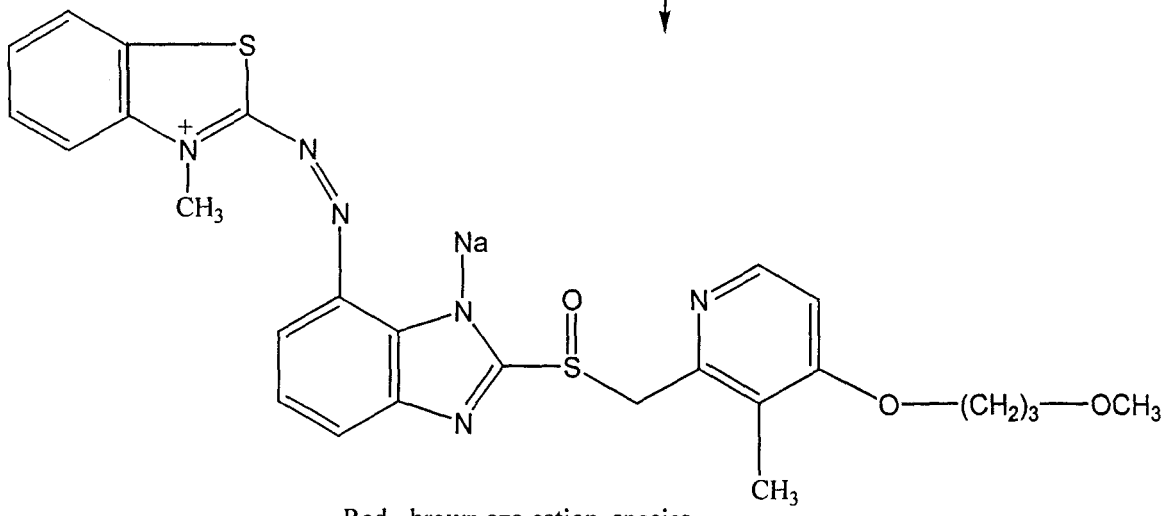


MBTH Electrophilic intermediate of MBTH

Step 2



Rabeprazole sodium



Red - brown azo cation species

Scheme 5.1

Meisenheimer complex which absorbs maximally at 420 nm. The blank consisting of CDNB in DMSO has a characteristic band at 353 nm **(Fig. 5.3).**

The stoichiometry was established by mole ratio method. The results are shown in **Fig 5.4**. It is apparent from the figure that the combining molar ratio between rabeprazole sodium and 1-chloro 2,4-dinitro benzene is 2:1. This stoichiometric ratio is comparable with the previous results showed by 1,3,5-trinitrophenyl piperidine complex. The reaction sequence is shown in **Scheme 5.2**.

Solution stability

The stability of rabeprazole sodium solution was ascertained in distilled water, DMSO and acetic acid. The reference drug and quality control sample solutions prepared in DMSO showed no change in the absorption spectra for 6 h at room temperature in DMSO, distilled water and 3.50×10^{-4} - 7.0×10^{-3} M acetic acid. A single spot was observed in reference drug and quality control sample solutions with R_f value of 0.68, which corresponds to the rabeprazole sodium. The reaction products of methods A and B were also examined by TLC, which indicated the R_f values of 0.45 and 0.58, respectively.

Optimization of variables

The optimization of variables for methods A and B was assessed by testing several parameters such as temperature, heating time, solvents, concentrations of ammonium cerium (IV) nitrate, MBTH and CDNB.

Method A

Effect of reaction time

The influence of the reaction time on the absorbance of the product was studied by taking 100 $\mu g\ ml^{-1}$ of rabeprazole sodium with 1.7 ml of 2×10^{-2} M ammonium cerium (IV) nitrate and 1.5 ml of 1.7×10^{-3} M MBTH in 10 ml volumetric

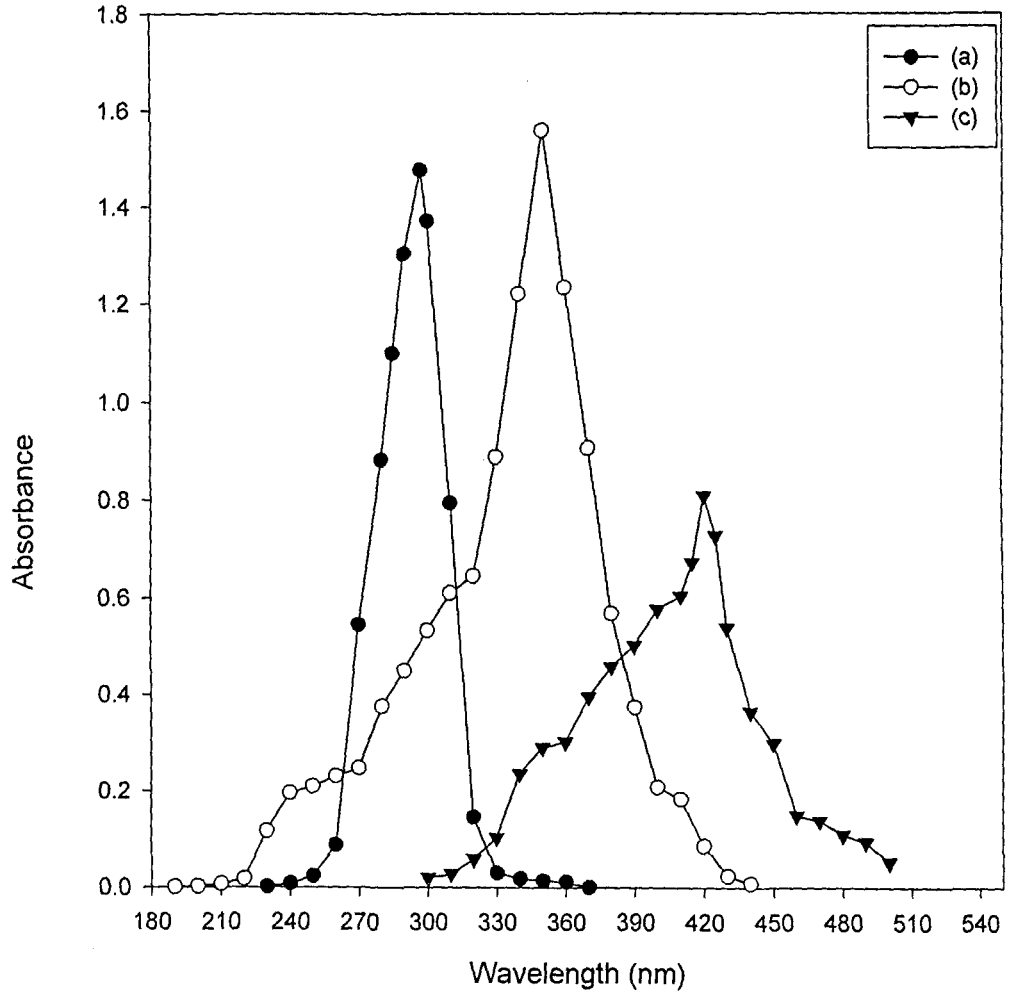


Fig. 5.3. Absorption spectra of (a) 75.0 μg ml^{-1} rabeprazole sodium in DMSO (b) blank solution: 1.18 $\times 10^{-2}$ M CDNB in DMSO (c) sample solution: 2.37 $\times 10^{-2}$ M CDNB + 150 μg ml^{-1} rabeprazole sodium in DMSO.

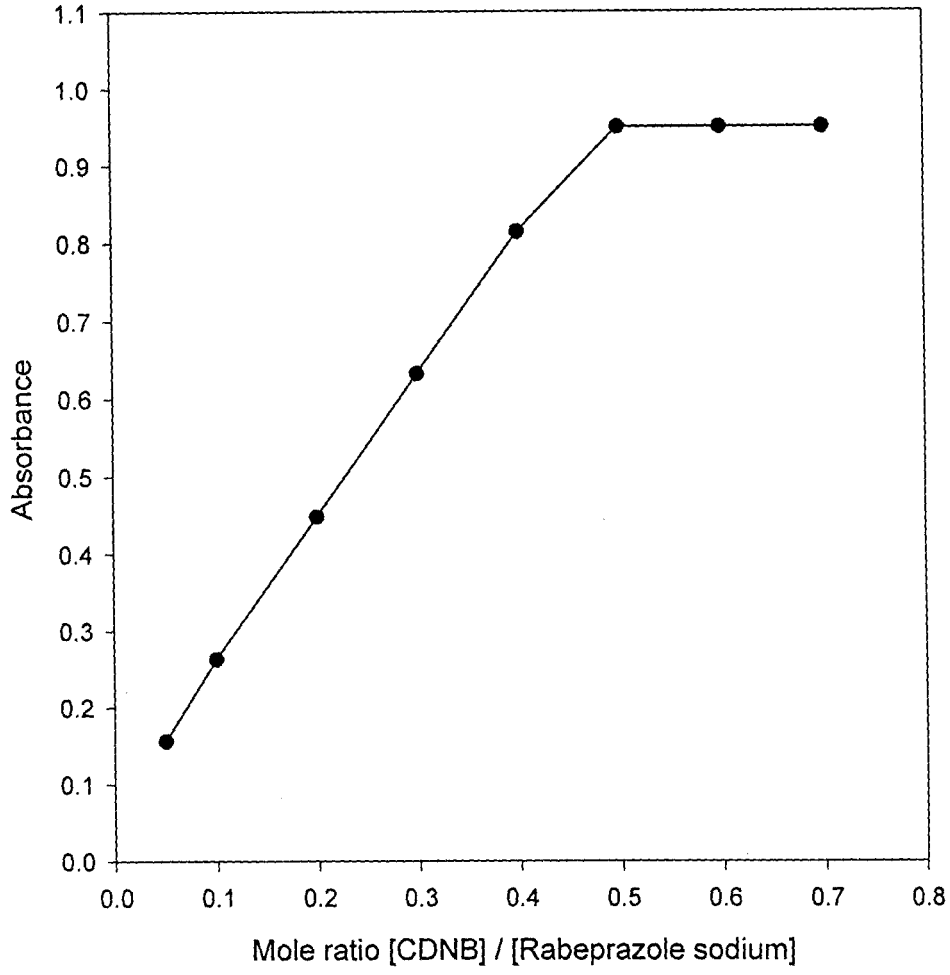
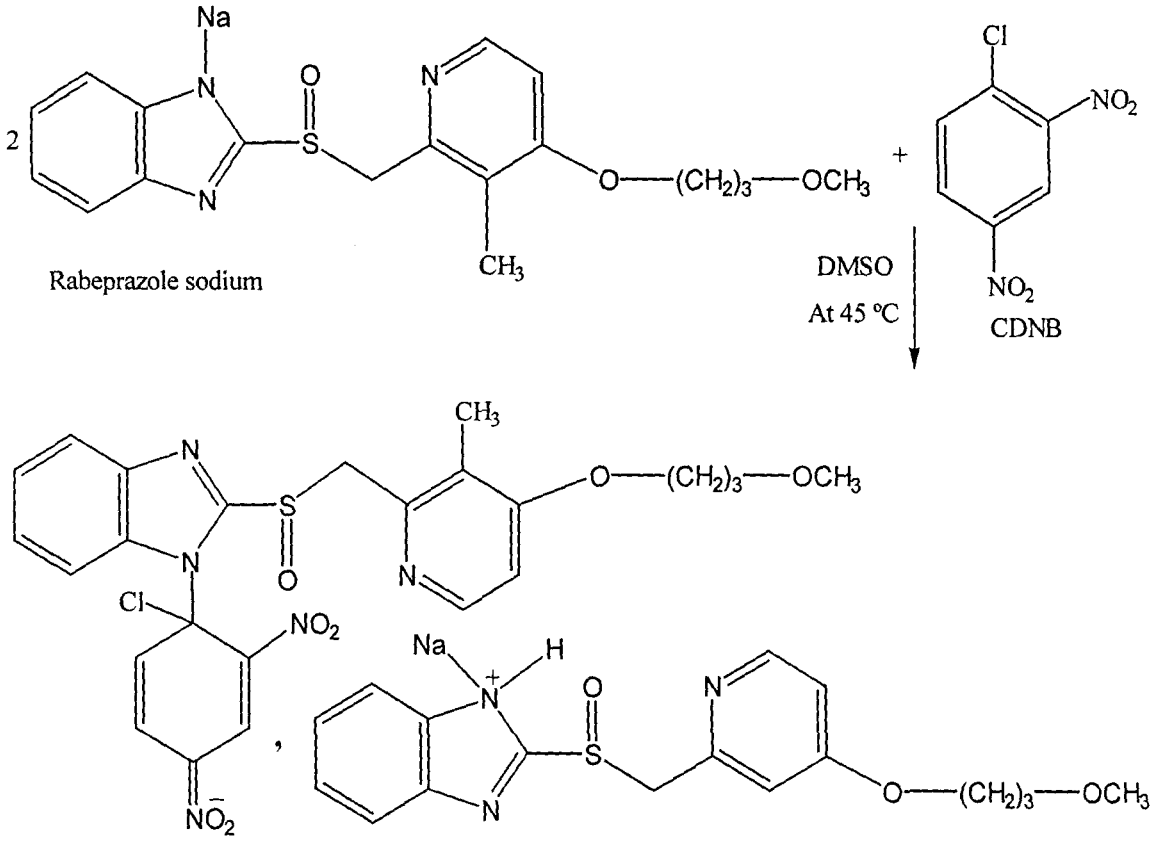


Fig. 5.4. Mole ratio plot for stoichiometric ratio between rabeprazole sodium and CDNB for method B.



Meisenheimer yellow complex

Scheme 5.2

flask. It was found that the maximum absorbance was achieved at 8 min of reaction and remains constant up to 12 min (**Fig. 5.5**). Therefore, a time of 10 min at room temperature was selected as an optimum reaction time.

Effect of the concentration of ammonium cerium (IV) nitrate

The influence of the concentration of ammonium cerium (IV) nitrate on the absorbance of the product was investigated in the range of 2.0×10^{-4} - 4.0×10^{-3} M. It was observed that the maximum absorbance was attained with 3.0×10^{-3} M ammonium cerium (IV) nitrate (**Fig. 5.6**) and remained constant up to 4.0×10^{-3} M. Therefore, 3.4×10^{-3} M ammonium cerium (IV) nitrate was taken as the optimum concentration for the determination process.

Effect of the concentration of MBTH

The effect of the concentration of MBTH on the absorbance of the product was studied in the range of 1.71×10^{-5} - 3.25×10^{-4} M. A constant absorbance was obtained with 1.88×10^{-4} M MBTH, beyond this further increase in the concentration of MBTH up to 3.25×10^{-4} M, resulted in no change in the absorbance (**Fig 5.7**). Thus, 3.25×10^{-4} M MBTH was adopted as an optimum concentration for the maximum absorbance in the determination procedure.

Method B

Effect of temperature and time

The effect of temperature on the reaction between rabeprazole sodium (150 μg ml^{-1}) and CDNB (2.84×10^{-2} M) was studied at 35, 40, 45 and 50°C. It was observed that the equilibrium was attained at 18, 14, 8 and 8 min at temperature of 35, 40, 45 and 50°C, respectively. To speedup the determination process and for the sake of good recovery results, optimum temperature of 45°C was chosen for the estimation of rabeprazole sodium. It was also observed that the absorbance at 45°C was

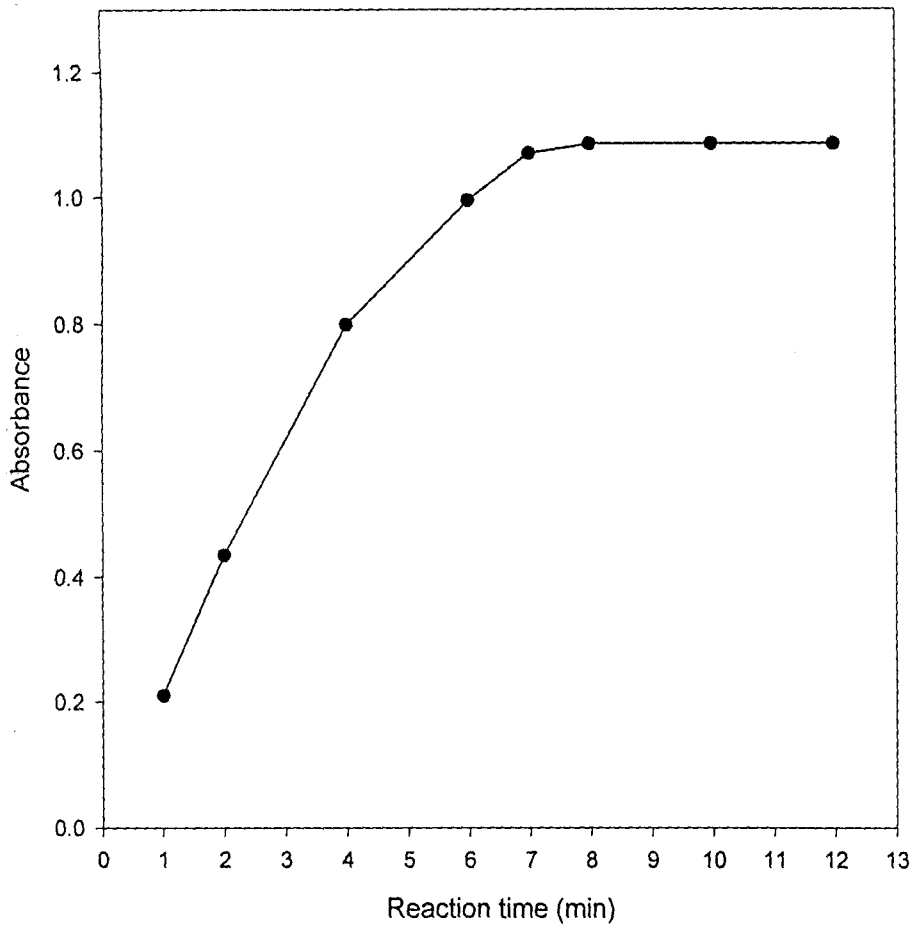


Fig. 5.5. Effect of time on the absorbance of colour reaction for Method A

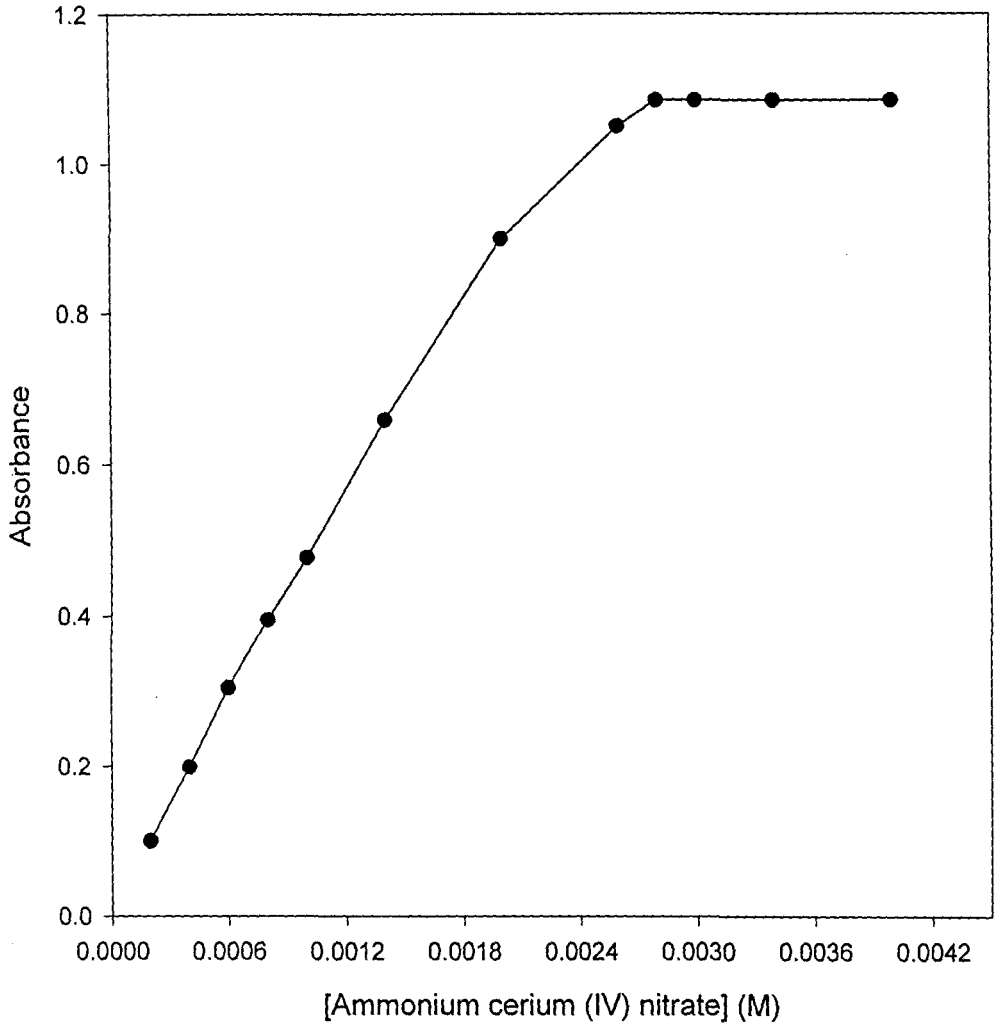


Fig. 5.6. Effect of the concentration of ammonium cerium (IV) nitrate on the absorbance of coloured complex (Method A).

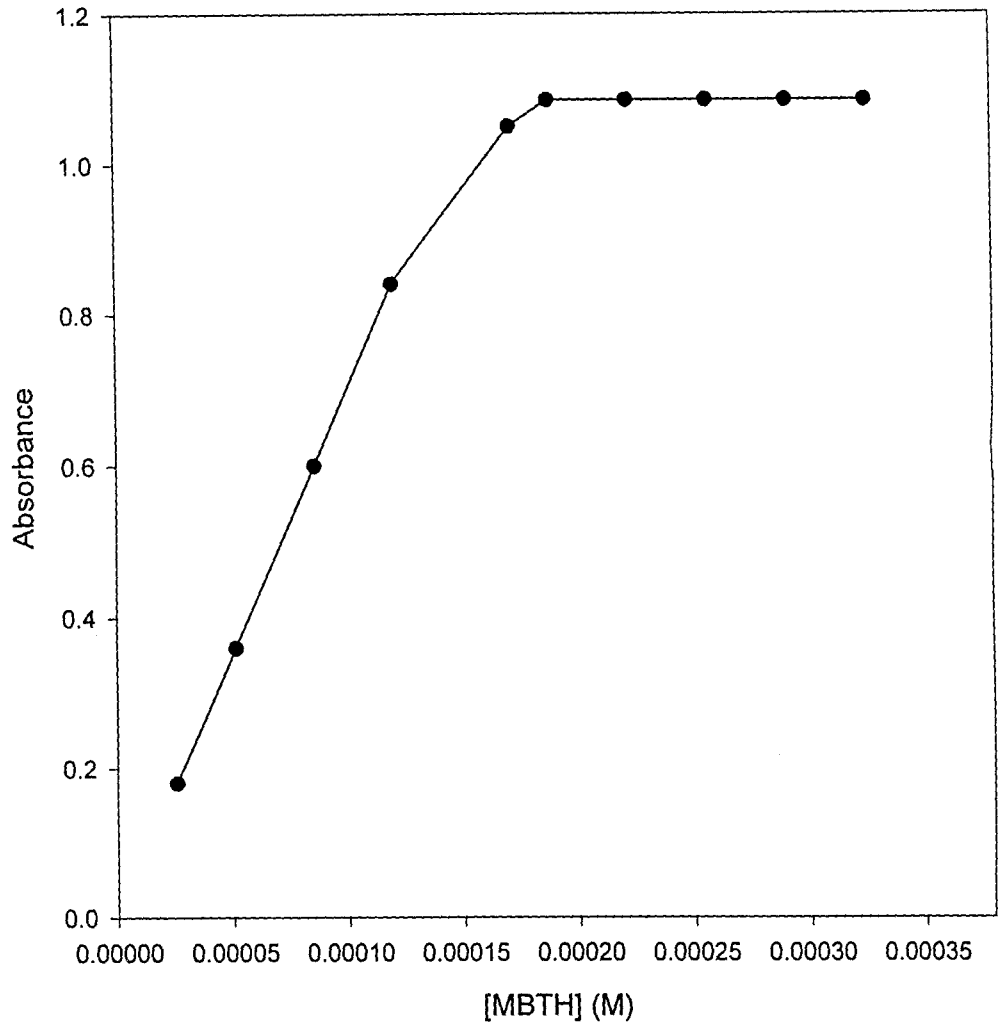


Fig. 5.7. Effect of the concentration of MBTH on the absorbance of coloured complex (Method A).

constant in the range of 8-12 min (**Fig 5.8**). Therefore, the optimum time of heating for the maximum absorbance was chosen to be 10 min for determination procedure.

Effect of the concentration of CDNB

The influence of CDNB concentration on the absorbance of yellow coloured complex was studied in the range of 1.18×10^{-3} - 3.32×10^{-2} M. It was found that the maximum absorbance was obtained in the range of 2.37×10^{-2} - 3.32×10^{-2} M CDNB (**Fig 5.9**). Therefore, the optimum concentration of 2.84×10^{-2} M CDNB was recommended for determination procedure.

Statistical performance of the analytical data

The calibration curves were constructed by plotting absorbance against concentration of rabeprazole sodium for the proposed methods. Beer's law was obeyed over the concentration ranges 14-140 μg ml^{-1} and 7.5-165 μg ml^{-1} with molar absorptivity of 4.104×10^3 l $mol^{-1}cm^{-1}$ and 2.069×10^3 l $mol^{-1}cm^{-1}$ for methods A and B, respectively. The calibration data were fitted to the equation, $A = a + bC$, where A is the absorbance at relevant λ_{max}; C is the concentration in μg ml^{-1}; b is the slope and a is the intercept of calibration. The regression parameters are summarized in **Table 5.1**. The high values of correlation coefficients (0.9999) for both methods indicated excellent linearity. In order to verify that the proposed methods are free from procedural error, the experimental intercept of the calibration lines were tested for significance of the deviation from the theoretical intercept as zero. For this justification, the values of t-calculated from the relation, $t = a / S_a$ [18] were found to be 0.246 and 1.596 for methods A and B, respectively, which did not exceed the theoretical t-value (2.365) at 95% confidence level. This indicated that the intercepts for methods A and B are not significantly different from zero.

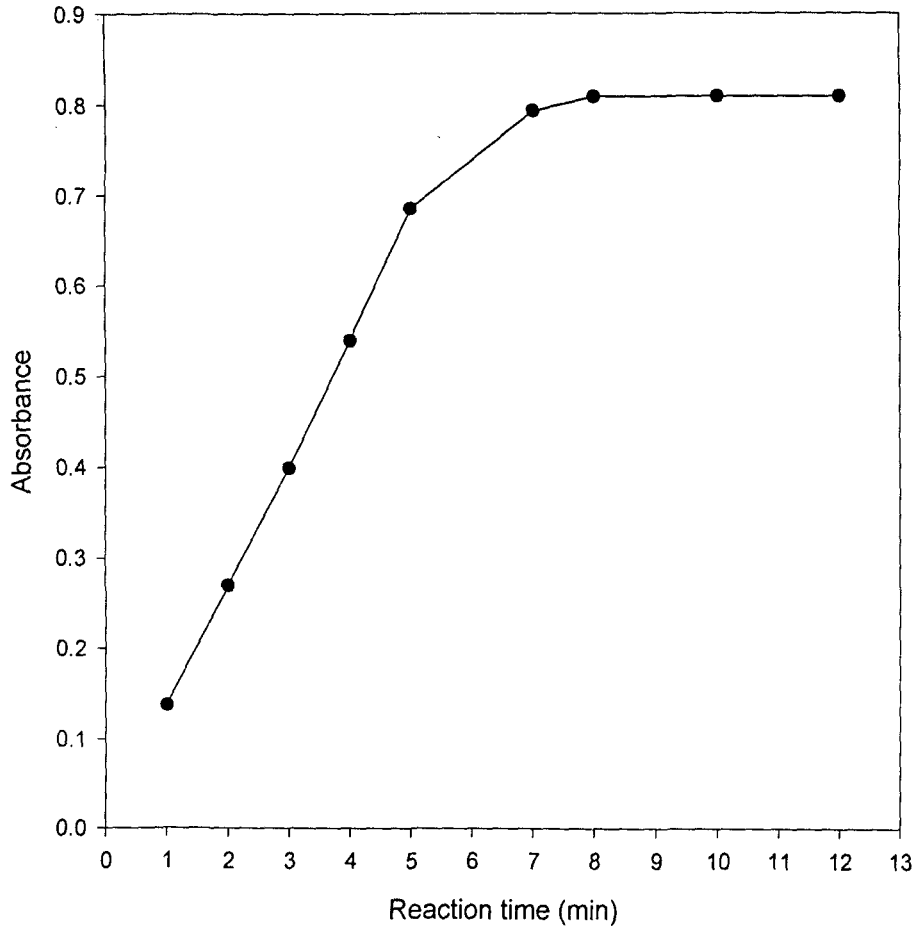


Fig. 5.8. Effect of time on the absorbance of colour reaction for Method B.

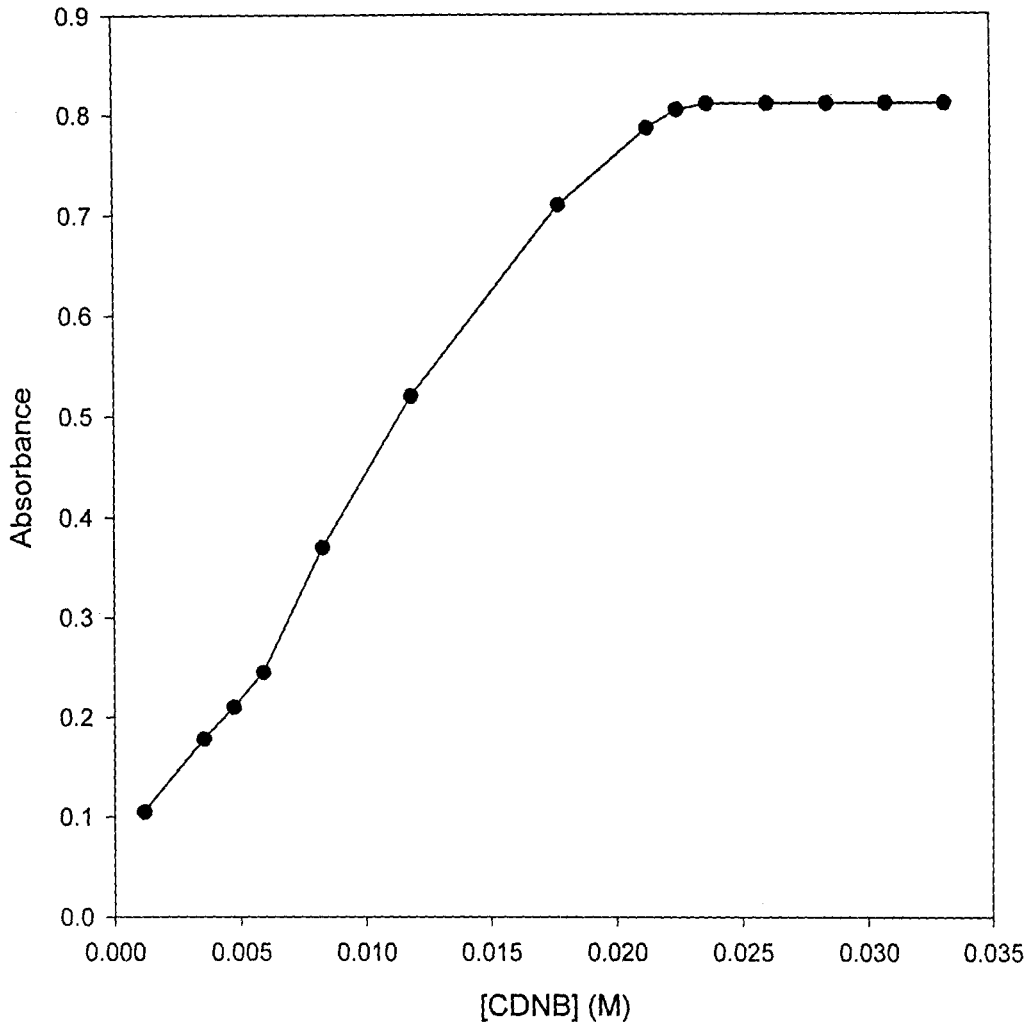


Fig. 5.9. Effect of the concentration of CDNB on the absorbance of yellow coloured complex (Method B).

Table 5.1
Summary of validation data for the determination of rabeprazole sodium

Parameters	Method A	Method B
Wavelength (nm)	475	420
Beer's law limit (μg ml^{-1})	14 - 140	7.5 - 165
Molar absorptivity (l $mol^{-1}cm^{-1}$)	4.104×10^3	2.069×10^3
Linear regression equation	$A = 6.041 \times 10^{-4} +$ $1.07 \times 10^{-2} C$	$A = 1.020 \times 10^{-3} +$ $5.0 \times 10^{-3} C$
±tSa[b)]	6.828×10^{-3}	1.774×10^{-3}
±tSb[b)]	9.065×10^{-5}	1.886×10^{-5}
Correlation coefficient (r)	0.9999	0.9999
Variance (S_0^2) of calibration line	1.989×10^{-5}	1.513×10^{-6}
Detection limit (μg ml^{-1})	1.38	0.75
Quantitation limit (μg ml^{-1})	4.18	2.27

Ruggedness and robustness

The ruggedness of methods A and B was evaluated by assaying the contents of rabeprazole sodium in tablet formulation using Spectronic $20D^+$ and Shimadzu UV 1240 spectrophotometers. The percent recoveries ± RSD resulted from Spectronic $20D^+$ spectrophotometer (100.02 ± 0.06 and 100.05 ± 0.09 for methods A and B, respectively) and Shimadzu UV 1240 (100.05 ± 0.06 and 100.04 ± 0.06 for methods A and B, respectively) were compared. The results agreed well within the acceptable limits with permissible bias.

The robustness of the methods A and B relative to each operational parameter was challenged. The operational parameters investigated were as follows:

Method A

- room temperature, 25 ± 1°C
- reaction time, 10 ± 2 min
- volume of 1.7×10^{-3} M MBTH, 1.5 ± 0.4 ml
- volume of 2.0×10^{-2} M ammonium cerium (IV) nitrate, 1.7 ± 0.3 ml

Method B

- heating temperature, 45 ± 1°C
- reaction time, 10 ± 2 min
- volume of 5.59×10^{-3} M CDNB, 2.4 ± 0.4 ml

The robustness of the proposed methods was assessed by analyzing active drug content in Rabicip-20. The quality control sample solution containing 80 μg ml^{-1} of the drug was analyzed five times using methods A and B. The percent recoveries ± RSD for methods A (100.02 ± 0.09) and B (100.05 ± 0.08) were found to be appreciable, thus indicated that the proposed methods are robust.

Specificity and selectivity

The proposed spectrophotometric conditions were found to be specific and selective in the presence of tablet excipients. It was observed that common excipients present in tablet formulations did not cause any significant interference.

Accuracy and precision

The intra day precision was evaluated by determining rabeprazole sodium at three concentration levels for five times within the same day (**Table 5.2**). As can be seen from **Table 5.2** that the percent relative error and relative standard deviation (%) were in the ranges of 0.014 - 0.571; 0.040 - 0.47 and 0.018 - 0.190; 0.040 - 0.45 for methods A and B, respectively. Also, the inter day precision was evaluated over a period of five days and the percent relative error and relative standard deviation (%) were found to be 0.007 - 0.429; 0.07 - 0.56 and 0.024 - 0.286; 0.040 - 0.45 for methods A and B, respectively.

The accuracy of the proposed methods A and B was ascertained by recovery studies using standard addition method. The results are summarized in **Table 5.3**. The mean recoveries and RSD for methods A and B were in the ranges 100.04 ± 0.065 - 100.16 ± 0.194 % and 100.01 ± 0.042 - 100.05 ± 0.109 %, respectively which can be considered to be very satisfactory.

The proposed procedures have been successfully applied to quantitate rabeprazole sodium in commercial dosage forms. The results obtained by methods A and B were compared with those of reference method in terms of mean recovery, RSD, θ_L, θ_U, t- and F- values (**Table 5.4**). It is evident from **Table 5.4** that the assay results showed good agreement between proposed methods and the reference method as t- and F- values were less than the theoretical ones at 95 % confidence level and θ_L and θ_U were less than ± 2.0 %. Therefore, it is concluded that the

Table 5.2
Summary of accuracy and precision results of the proposed methods

Proposed methods	Concentration ($\mu g\ ml^{-1}$)		RSD[a] (%)	R.E.[b] (%)	SAE[b]	C.L.[c]
	Taken	Found ± SD				
Method A						
Intra day assay	14.0	14.08 ± 0.07	0.47	0.571	0.029	0.082
	60.0	60.08 ± 0.08	0.13	0.133	0.035	0.073
	140.0	139.98 ± 0.05	0.04	0.014	0.023	0.097
Inter day assay	14.0	14.06 ± 0.08	0.56	0.429	0.035	0.097
	60.0	60.00 ± 0.08	0.14	0.016	0.036	0.101
	140.0	140.01 ± 0.09	0.07	0.007	0.042	0.116
Method B						
Intra day assay	10.5	10.52 ± 0.05	0.45	0.190	0.021	0.059
	75.0	75.05 ± 0.08	0.11	0.066	0.035	0.098
	165.0	165.03 ± 0.07	0.04	0.018	0.029	0.080
Inter day assay	10.5	10.53 ± 0.05	0.45	0.286	0.022	0.058
	75.0	75.04 ± 0.08	0.11	0.053	0.037	0.103
	165.0	165.04 ± 0.07	0.04	0.024	0.031	0.087

[a] Mean for five independent analyses.
[b] R.E. and SAE indicate relative error (%) and standard analytical error.
[c] C.L. is the confidence limit at 95% confidence level and four degrees of freedom (t = 2.776).

Table 5.3
Summary of results evaluated by standard addition technique for the quantitation of rabeprazole sodium in pharmaceutical formulations

Formulations	Amount ($\mu g\ ml^{-1}$)		Found ± SD[a]	Recovery ± RSD[a]	SAE	C.L.
	Taken	Added				
Method A						
Rabicip-20 (Cipla)	40	40	80.13 ± 0.13	100.16 ± 0.156	0.056	0.155
	40	80	120.07 ± 0.09	100.06 ± 0.077	0.042	0.116
Rablet-20 (Lupin)	40	40	80.11 ± 0.16	100.13 ± 0.194	0.069	0.193
	40	80	120.05 ± 0.12	100.04 ± 0.102	0.054	0.152
Rapeed-20 (Alkem)	40	40	80.03 ± 0.16	100.09 ± 0.194	0.070	0.193
	40	80	120.05 ± 0.08	100.04 ± 0.065	0.035	0.097
Method B						
Rabicip-20 (Cipla)	45	45	90.05 ± 0.09	100.04 ± 0.096	0.039	0.108
	45	90	135.04 ± 0.07	100.03 ± 0.048	0.029	0.081
Rablet-20 (Lupin)	45	45	90.04 ± 0.09	100.05 ± 0.099	0.089	0.247
	45	90	135.05 ± 0.06	100.03 ± 0.042	0.025	0.072
Rapeed-20 (Alkem)	45	45	90.02 ± 0.10	100.03 ± 0.109	0.044	0.123
	45	90	135.01 ± 0.06	100.01 ± 0.045	0.027	0.075

[a]Mean for five independent analyses

Table 5.4
Summary of comparison results of the proposed methods with the reference method at 95% confidence level

Formulations	Method A				Method B				Reference method	
	Recovery (%)	RSD[a] (%)	t- & F [b]	θ_L & θ_U [c]	Recovery (%)	RSD[a] (%)	t- & F [b]	θ_L & θ_U [c]	Recovery (%)	RSD[a] (%)
Rabicip-20 (Cipla)	100.04	0.064	t = 0.052 F = 1.162	θ_L = 0.987 θ_U = 1.012	100.04	0.065	t = 0.053 F = 1.153	θ_L = 0.990 θ_U = 1.010	100.06	0.068
Rablet-20 (Lupin)	100.02	0.094	t = 0.246 F = 1.132	θ_L = 0.992 θ_U = 1.009	100.05	0.088	t = 0.133 F = 1.008	θ_L = 0.989 θ_U = 1.012	99.99	0.087
Rapeed-20 (Alkem)	100.05	0.064	t = 0.098 F = 1.158	θ_L = 0.988 θ_U = 1.013	100.04	0.064	t = 0.052 F = 1.163	θ_L = 0.987 θ_U = 1.012	100.06	0.069

[a]Mean for five independent analyses
[b]Theoretical t-value (ν =8) and F-value (ν =4,4) at 95% confidence level are 2.306 and 6.39, respectively.
[c]θ_L = 0.98 and θ_U = 1.02 are acceptable bias, based on recovery experiments and are within ±2%.

proposed methods A and B are applicable for routine quality control analysis of rabeprazole sodium in commercial dosage forms with acceptable recovery results less than ± 2.0 %.

CONCLUSION

The proposed methods provide simple, accurate and reproducible quantitative analysis for the assay of rabeprazole sodium in commercial dosage forms. Both methods are specific and selective. In addition, the proposed methods have high molar absorptivity (4.1×10^3 l mol^{-1} cm^{-1} for method A and 2.07×10^3 l mol^{-1} cm^{-1} for method B) with broad linear dynamic range (14 - 140 μg ml^{-1} for method A and 7.5 - 165 μg ml^{-1} for method B) and high tolerance limit for excipients found in dosage forms. Therefore the proposed methods are recommended for the routine quality control analysis of rabeprazole sodium in commercial dosage forms.

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Kinetic Spectrophotometric Analysis of Pantoprazole in Commercial Dosage Forms

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A kinetic spectrophotometric method has been developed which is based on the oxidation of pantoprazole with Fe(III) in sulfuric acid medium. Fe(III) subsequently reduces to Fe(II), which is coupled with potassium ferricyanide to form Prussian blue. The reaction is followed spectrophotometrically by measuring the increase in absorbance with time (1 - 8 min) at 725 nm. The initial rate method is adopted for constructing the calibration graph, which is linear in the concentration range of 5 - 90 μg ml^{-1}. The regression analysis yields the calibration equation, $v = 3.467 \times 10^{-6} + 4.356 \times 10^{-5}C$. The limits of detection and quantitation are 1.46 and 4.43 μg ml^{-1}, respectively. The proposed method was optimized and validated both statistically and through recovery studies. The experimental true bias of all samples is $< \pm 2.0\%$. The method has been successfully applied to the determination of pantoprazole in pharmaceutical preparations.

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Introduction

Pantoprazole, 5-difluoromethoxybenzimidazole-2-yl 3,4-dimethoxy-2-pyridylmethyl sulfoxide (CAS, 102625-70-7; MW, 383.4) is an irreversible proton pump (H^+/K^+-ATPase) inhibitor (PPI) that decreases acid secretion from gastric parietal cells; its major metabolite is pantoprazole sulfone.[1] The structural formulae are shown in Scheme 1. It is also effective in Zollinger-Ellison syndrome and in preventing ulcer rebleeding. Thus pantoprazole is a valuable alternate to other PPIs in the treatment of acid-related disorders. The drug is officially listed in Martindale The Extra Pharmacopoeia.[2] The assay of drug in bulk and dosage forms is not announced in any pharmacopoeia and therefore requires much more investigation in order to assure the exact quantity of drug in pharmaceutical formulations. A literature survey reveals that high performance liquid chromatography,[3-5] capillary zone electrophoresis,[6] and voltammetry[7] have been employed for its quantification. The above-mentioned techniques, of course, are sensitive enough but are expensive. Spectrophotometry is the technique of choice even today due to its inherent simplicity. It is frequently used in the laboratories of the developing countries to overcome a variety of analytical problems. In the literature only a few spectrophotometric methods have been reported. Two derivative spectrophometric procedures[8,9] have been described for the determination of pantoprazole in drug formulations. The drug content in pharmaceutical preparations has been determined spectrophotometrically[10] in the visible region based on the reaction of drug with Fe(III) to form an orange-colored chelate, which absorbed maximally at 455 nm. The drug forms a ternary complex with eosin and copper, which is a basis for its analysis at 549 nm.[11] The charge transfer complexation reaction between the drug and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone or iodine has been used to quantify the drug spectrophotometrically.[11]

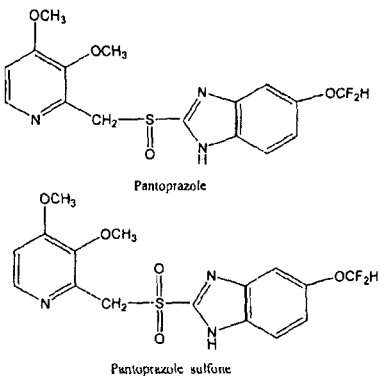
A kinetic spectrophotometric method based on the reaction of pantoprazole with 1-fluoro-2,4-dinitrobenzene in dimethyl sulfoxide medium[12] has been reported; this showed a linear response over the concentration range of 10.0 - 20.0 μg ml^{-1}.

In this paper, a kinetic spectrophotometric method for the determination of pantoprazole in drug formulations is described. The method is based on the oxidation of pantoprazole with Fe(III) in sulfuric acid medium; Fe(III) subsequently reduced to Fe(II), which reacts with potassium ferricyanide to form a Prussian blue product, absorbing maximally at 725 nm. The initial-rate method is applied for the determination of pantoprazole. The method is optimized and validated as per the guidelines of the International Conference on Harmonisation.[13]

Experimental

Apparatus

A Shimadzu UV-visible spectrophotometer (UVmini-1240, Shimadzu Corporation, Kyoto, Japan) was used for all absorbance measurements with matched quartz cells.



Scheme 1

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Materials and reagents

All chemicals and reagents were of analytical or pharmaceutical grade. Pantoprazole was kindly supplied by Concept Pharma Ltd. (Mumbai, India) and was used as the reference standard. A standard solution of pantoprazole (0.1%) was prepared by dissolving 50 mg in 50 ml distilled water. Pharmaceutical formulations of pantoprazole such as Pantec 20 (Concept, Mumbai, India) and Pantodac 20 (Zydus Cadila, Ahmedabad, India) were purchased from local markets.

Ammonium ferric sulfate (3.0×10^{-3} M; Fluka Chemie AG, Switzerland) solution was prepared by dissolving 144.66 mg of ammonium ferric sulfate in 0.003 M sulfuric acid. Potassium ferricyanide (2.5×10^{-3} M; Fluka Chemie AG, Switzerland) solution was prepared in distilled water.

Recommended procedure

Aliquots of 0.05 - 0.9 ml standard solutions of pantoprazole were pipetted into a series of 10.0 ml standard volumetric flasks. To each flask, 1.7 ml of 3.0×10^{-3} M ammonium ferric sulfate was added, followed by 1.5 ml of 2.5×10^{-3} M potassium ferricyanide. Then the mixture was diluted to volume with distilled water at 35 ± 1°C. The contents of each flask were mixed well and then immediately transferred to the spectrophotometric cell. The increase in absorbance was recorded at 725 nm as a function of time for 8.0 min. The initial rate of the reaction (v) at different concentrations was obtained from the slope of the initial tangent to the absorbance-time curve. The calibration graph was constructed by plotting the initial rate of reaction, v *versus* concentration of pantoprazole (C, μg ml^{-1}). The amount of the drug was evaluated either from the calibration graph or from the regression equation.

Procedure for the determination of pantoprazole in pharmaceutical formulations

The quality control sample solution containing pantoprazole at a concentration of 1.0 mg ml^{-1} was prepared. The contents of 5 tablets of 20 mg strength were obtained by gentle peeling of the hard coated shells. The contents of the tablets were put in distilled water and left for 10.0 min for complete dispersion of the drug. Then the solution was filtered through a piece of Whatmann No. 42 filter paper (Whatmann International Limited, Kent, UK) in a 100 ml standard volumetric flask. The residue was washed well with distilled water for complete recovery of the drug and then the mixture was diluted up to the mark with distilled water. The assay was completed following the proposed procedure for the determination of pantoprazole.

Procedure for the determination of pantoprazole in synthetic mixtures

Synthetic mixtures of pantoprazole were prepared by taking various excipients commonly used in tablet dosage forms with 800 μg of pantoprazole in 10 ml standard volumetric flask and tested to study the interferences of excipients such as sodium stearyl fumarate, magnesium stearate, corn starch, lactose and talc.

Procedure for reference method[11]

Aliquots of 0.1 - 0.6 ml of 0.1% pantoprazole were pipetted into a series of 10 ml standard volumetric flasks. To each flask, 6.0 ml of 0.4% 2,3-dichloro-5,6-dicyano-1,4-benzoquinone was added and the mixture was diluted to volume with acetonitrile. The absorbance was measured against the reagent blank at 457 nm. The amount of the drug in a given sample was computed from the calibration graph.

Validation

The proposed kinetic method has been validated for specificity, linearity, limit of detection, precision, accuracy and recovery.

Specificity. The reference standard and quality control samples of pantoprazole were subjected to stress conditions of light, heat, acid, base and oxidants. Each stressed sample was measured to determine the content of pantoprazole and the results were compared to those for an unstressed time zero reference solution. The reference assay value for each unstressed product was evaluated and the contents of degradation in the stressed and control samples were estimated relative to this assay value.

Linearity. The linearity was evaluated with 10 standard solutions: 5.0, 10.0, 15.0, 20.0, 35.0, 50.0, 60.0, 70.0, 80.0 and 90.0 μg ml^{-1}. The determination was repeated five times at each concentration level.

Limit of detection (LOD) and quantitation (LOQ). The limits of detection (LOD) and quantitation (LOQ) for the assay were calculated using the equations:[14]

$$\mathrm{LOD} = 3.3 \times S_0/b \text{ and } \mathrm{LOQ} = 10 \times S_0/b$$

where S_0 and b are the standard deviation and the slope of the calibration line.

Precision and accuracy. Intra-day precision and accuracy of the proposed method were evaluated by replicate analysis (n = 5) of calibration standards at three concentration levels (20.0, 50.0, and 80.0 μg ml^{-1}). Inter-day precision and accuracy were determined by assaying the calibration standards at the same concentration levels on five consecutive days. Precision and accuracy were based on the calculated relative standard deviation (RSD, %) and relative error (RE, %) of the found concentration compared to the theoretical one, respectively.

Recovery studies. The recovery of pantoprazole from commercial dosage forms was estimated by the standard addition method. For this purpose, a volume of 2.0 ml (or 5.0 ml) of sample solution was spiked with 2.0, 4.0, 6.0 and 7.0 ml (or 1.0, 2.0, 3.0 and 4.0) of reference standard solution (1.0 mg ml^{-1}) in a 100 ml standard volumetric flask and the mixture was diluted up to the mark with distilled water. Each level was repeated 5 times. The nominal value was determined by the proposed procedure.

Evaluation of bias. The point and interval hypothesis tests have been performed to compare the results of the proposed method with those of the reference method at 95% confidence level. The bias was evaluated by an interval hypothesis test based on the mean values of the proposed method (method 1) and the reference method (method 2). The test method is considered acceptable when its true mean is within ±2.0% of that of the reference method. This can be written as

$$0.98 < \mu_2/\mu_1 < 1.02$$

which can be generalized to

$$\theta_L < \mu_2/\mu_1 < \theta_U$$

where θ_L and θ_U are lower and upper acceptance limits, respectively, which were calculated from the following quadratic equation:[15]

$$\theta^2\left(\overline{x_1^2} - S_p^2 t_{tab}^2/n_1\right) + \theta\left(-2\,\overline{x_1 x_2}\right) + \theta^2\left(\overline{x_2^2} - S_p^2 t_{tab}^2/n_2\right) = 0$$

Results and Discussion

Dialkyl/diaryl/alkyl-aryl sulfoxides undergo oxidation with a number of oxidants[16,17] to form sulfone derivatives. Pantoprazole is a sulfoxide derivative that is oxidized in a similar manner by ammonium ferric sulfate in moderately acidic medium to form pantoprazole sulfone,[3] and itself reduces to Fe(II). The reduced Fe(II) immediately reacts with potassium ferricyanide, resulting in the formation of Prussian blue product,[18] which absorbs maximally at 725 nm. The absorbance of the colored solution increases with time and hence, a kinetically-based spectrophotometric method was elaborated to assay the pantoprazole in pharmaceutical formulations. The various experimental parameters affecting the formation of colored product were optimized and used throughout the experiment.

TLC study of pantoprazole and its oxidized product

To identify pantoprazole and pantoprazole sulfone, we used thin layer chromatography. The reference standard solutions of pantoprazole or of pantoprazole sulfone was applied on TLC plates coated with silica gel G (Merck, India) and developed in chloroform-methanol (10:0.7 v/v) solvent system. The plates were free from mobile phase, dried and spots were detected in iodine chamber. The R_f values were 0.54 and 0.66 for pantoprazole and pantoprazole sulfone, respectively. The R_f value (0.66) of the reaction product confirmed the presence of pantoprazole sulfone.

Optimization of variables

The optimum conditions for the proposed method responsible for the formation of the blue product were studied and maintained throughout the experiment.

Effect of temperature. The effect of temperature on the initial rate of reaction was studied at 303, 308, 313 and 318 K. The absorbance-time curves showed that the reaction rate increases with increase in temperature. At temperature > 313 K, the linear dynamic range of determination decreases. The linear dynamic range, regression equation and correlation coefficient obtained at different temperatures are summarized in Table 1. The best linearity was obtained at 308 K and hence this temperature was selected as an optimum temperature for the determination process.

Effect of ammonium ferric sulfate concentration. The effect of ammonium ferric sulfate concentration on the initial rate of reaction (v) was studied in the range of 2.40×10^{-4} - 6.60×10^{-4} M keeping constant [potassium ferricyanide] = 3.75×10^{-4} M and [pantoprazole] = 2.09×10^{-4} M. The initial rate of reaction increased with increase in the concentration of ammonium ferric sulfate and became constant at 3.90×10^{-4} M, and remained as such up to 6.60×10^{-4} M. The results are summarized in Table 2. Therefore, a concentration of 5.10×10^{-4} M ammonium ferric sulfate was recommended for the determination procedure.

Effect of potassium ferricyanide concentration. The effect of potassium ferricyanide concentration on the initial rate of reaction (v) was investigated in the range of 2.50×10^{-5} - 5.00×10^{-4} M. The maximum value of the initial rate of reaction was obtained with 2.25×10^{-4} M potassium ferricyanide, after which further increase in the concentration of potassium ferricyanide up to 5.00×10^{-4} M resulted in no change in the initial rate of reaction (Table 2). Thus, the concentration of 3.75×10^{-4} M potassium ferricyanide was found to be most suitable concentration for the determination process.

Analytical data and method validation

The oxidation of pantoprazole takes place with Fe(III) in acidic medium, resulting in the formation of pantoprazole sulfone; subsequently, reduced Fe(II) reacts with potassium ferricyanide to form the blue product. The course of the reaction was followed spectrophotometrically at 725 nm. The initial rates of reaction for different concentrations of pantoprazole at 308 K were determined from the slopes of the initial tangent to the absorbance-time curves (Fig. 1); these rates are summarized in Table 3. The kinetic equation for the reaction of pantoprazole with Fe(III) and potassium ferricyanide

Table 1 Linear dynamic range, regression equation and correlation coefficient at different temperatures

Temp./K	Beer's law range/ μg ml^{-1}	Regression equation	Correlation coefficient, r
303	10.0 - 90.0 $n = 9$	$v = -4.615 \times 10^{-6} + 1.548 \times 10^{-5}C$	0.9993
308	5.0 - 90.0 $n = 10$	$v = 3.467 \times 10^{-6} + 4.356 \times 10^{-5}C$	0.9999
313	5.0 - 90.0 $n = 10$	$v = 2.333 \times 10^{-5} + 5.606 \times 10^{-5}C$	0.9995
318	5.0 - 70.0 $n = 8$	$v = 2.455 \times 10^{-5} + 6.559 \times 10^{-5}C$	0.9998

Table 2 Effect of the concentrations of ammonium ferric sulfate and potassium ferricyanide on the initial rate of reaction at [pantoprazole] = 2.086×10^{-4} M

Ammonium ferric sulfate[a]		Potassium ferricyanide[b]	
Concentration, C/mol l^{-1}	Initial rate of reaction, v/mol l^{-1} s^{-1}	Concentration, C/mol l^{-1}	Initial rate of reaction, v/mol l^{-1} s^{-1}
2.40×10^{-4}	2.98×10^{-4}	2.50×10^{-5}	1.07×10^{-4}
3.00×10^{-4}	5.95×10^{-4}	5.00×10^{-5}	2.52×10^{-4}
3.60×10^{-4}	8.77×10^{-4}	1.00×10^{-4}	4.27×10^{-4}
3.90×10^{-4}	1.24×10^{-3}	1.25×10^{-4}	6.66×10^{-4}
4.50×10^{-4}	1.24×10^{-3}	1.75×10^{-4}	1.15×10^{-3}
5.10×10^{-4}	1.24×10^{-3}	2.25×10^{-4}	1.24×10^{-3}
6.60×10^{-4}	1.24×10^{-3}	2.75×10^{-4}	1.24×10^{-3}
		3.25×10^{-4}	1.24×10^{-3}
		3.75×10^{-4}	1.24×10^{-3}
		5.00×10^{-4}	1.24×10^{-3}

a. Keeping constant [potassium ferricyanide] = 3.75×10^{-4} M.
b. Keeping constant [ammonium ferric sulfate] = 5.10×10^{-4} M.

Table 3 Initial rate of reaction for different concentrations of pantoprazole with [Fe(III)] = 5.10×10^{-4} M and [potassium ferricyanide] = 3.75×10^{-4} M at 308 K

Pantoprazole		Initial rate of reaction, v/ μg ml^{-1} s^{-1}
μg ml^{-1}	mol l^{-1}	
5.0	1.304×10^{-5}	2.166×10^{-4}
10.0	2.608×10^{-5}	4.416×10^{-4}
15.0	3.912×10^{-5}	6.583×10^{-4}
20.0	5.216×10^{-5}	8.750×10^{-4}
35.0	9.128×10^{-5}	1.520×10^{-3}
50.0	1.304×10^{-4}	2.170×10^{-3}
60.0	1.564×10^{-4}	2.660×10^{-3}
70.0	1.825×10^{-4}	3.050×10^{-3}
80.0	2.087×10^{-4}	3.500×10^{-3}
90.0	2.347×10^{-4}	3.900×10^{-3}

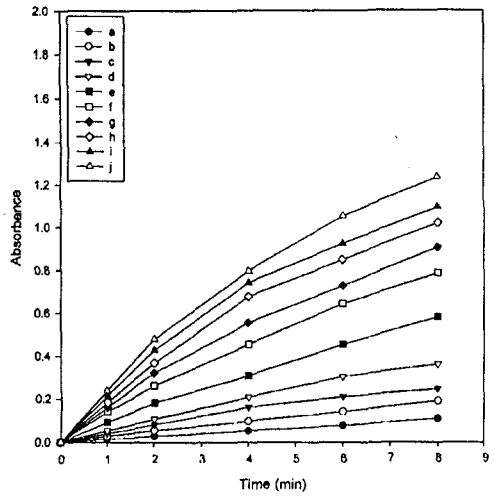

Fig. 1 Absorbance-time curves for the initial rate of reaction of pantoprazole: 5.10×10^{-4} M ammonium ferric sulfate and 3.75×10^{-4} M potassium ferricyanide with (a) 5.0, (b) 10.0, (c) 15.0, (d) 20.0, (e) 35.0, (f) 50.0, (g) 60.0, (h) 70.0, (i) 80.0 and (j) 90.0 μg ml^{-1} of pantoprazole.

is written as:

$$v = \frac{dx}{dt} = k_1 C^n_{Drug} C^m_{Fe(III)} C^l_{ferricyanide} \quad (1)$$

Under the optimized experimental conditions, *i.e.* $C_{Fe(III)} \geq 5.10 \times 10^{-4}$ M and $C_{ferricyanide} \geq 3.75 \times 10^{-4}$ M, the reaction became *pseudo*-zero order with respect to the reagent concentrations.

Therefore, the above equation reduced to $v = k_1 C^n_{Drug}$ where k_1 is the first rate constant and n is the order of the reaction. The order with respect to pantoprazole was evaluated from the plot of log v *versus* log C and was found to be 1.

Hence, the reaction would obey the *pseudo*-first order condition and thus the above equation reduced to $v = k_1 C_{Drug}$.

A calibration graph was constructed by plotting the initial rate of reaction (v) *versus* the pantoprazole concentration (C); the graph showed a linear relationship over the concentration range of 5.0 - 90.0 μg ml^{-1} at 308 K. The regression of initial rate *versus* C gave a linear regression equation, $v = 3.467 \times 10^{-6} + 4.356 \times 10^{-5}C$ with coefficient of correlation, $r = 0.9999$. The confidence limits for the slope of the line of regression and intercept were computed using the relation $b \pm tS_b$ and $a \pm tS_a$ at 95% confidence level and were found to be $4.356 \times 10^{-5} \pm 4.806 \times 10^{-7}$ and $3.467 \times 10^{-6} \pm 2.519 \times 10^{-5}$, respectively. The values of confidence limits for slope and intercept indicated the high reproducibility of the initial rate method. The limits of detection (LOD) and quantitation (LOQ) were found to be 1.46 and 4.43 μg ml^{-1}, respectively. The small value of variance (3.716×10^{-10} μg ml^{-1}) also confirmed the negligible scattering of the calibration data points around the line of regression.

Solution stability and selectivity

Pantoprazole is stable under neutral to moderately acidic conditions (pH ~3.5 - 7.4).[19] The solution stability of pantoprazole was checked by observing UV spectra of pantoprazole for 14 h. The aqueous solution of the drug having λ_{max} at 298 nm showed no changes in the absorption spectra of standard and quality control sample solutions of drug for at least 14 h, when the solutions were stored at a temperature < 45°C.

Table 4 Test of precision (intra and inter day assays) for analyses of pantoprazole

Proposed method	Concentration/μg ml^{-1}		RSD[a], %	SAE[a]	C.L.[c]
	Taken	Found × SD[a]			
Intra-day assay	20.0	20.01 ± 0.05	0.24	0.021	0.059
	50.0	49.95 ± 0.09	0.18	0.041	0.113
	80.0	80.00 ± 0.08	0.09	0.033	0.093
Inter-day assay	20.0	19.95 ± 0.15	0.78	0.069	0.192
	50.0	49.95 ± 0.09	0.19	0.041	0.116
	80.0	80.01 ± 0.16	0.20	0.071	0.198

a. Mean for 5 independent analyses.
b. SAE, standard analytical error.
c. C.L., confidence limit at 95% confidence level and 4 degrees of freedom ($t = 2.776$).

To identify pantoprazole and pantoprazole sulfone, we used thin layer chromatography. The standard solution, quality control sample solution and oxidized product of pantoprazole were applied on TLC plates coated with silica gel and developed in chloroform-methanol (10:0.7 v/v) solvent system. The plates were air-dried and spots were detected in the iodine chamber. In the case of standard and quality control sample solutions, a single spot was observed with $R_f = 0.54$ corresponding to pantoprazole, whereas the oxidized product showed one spot with R_f value of 0.66, confirming the presence of pantoprazole sulfone. The proposed kinetic method is a selective one, since the major metabolite of pantoprazole *i.e.* pantoprazole sulfone does not interfere with the determination process.

Robustness

The robustness of the proposed method was investigated by challenging each operational parameter such as: 1.7 ml of 0.003 M ammonium ferric sulfate in 0.003 M H_2SO_4 (±0.4 ml); 1.5 ml of 0.0025 M potassium ferricyanide (±0.5 ml); 35°C (308 K) as the working temperature (±1°C).

Under these conditions quality control sample solutions from two commercial dosage forms claiminig 60.0 μg ml^{-1} of active pantoprazole were assayed by performing 5 independent analyses following the proposed kinetic method. The recovery results are appreciable with low values of standard deviation and relative standard deviations.

Accuracy and precision

The accuracy and precision of the proposed kinetic method were established by performing 5 independent analyses of pantoprazole in pure form at three different concentration levels (20, 50 and 80 μg ml^{-1}) by intra-day and inter-day precisions. The recovery results with relative standard deviation, standard analytical error and confidence limit are summarized in Table 4; they confirm that the accuracy and precision were acceptable. Thus the proposed method is effective for the estimation of pantoprazole.

The accuracy of the proposed kinetic method was also tested by performing recovery experiments through the standard addition method. The recovery was evaluated either by dividing the intercept by the slope value of the line of linear regression of the standard addition method or by the extrapolation of the same line of best fit (Figs. 2 and 3, Table 5). It is evident from Table 5 that the linearity of the regression line of the standard addition method was good. The attractive feature of the method is its relative freedom from interference by the usual tablet diluents and excipients in amounts far in excess of their normal

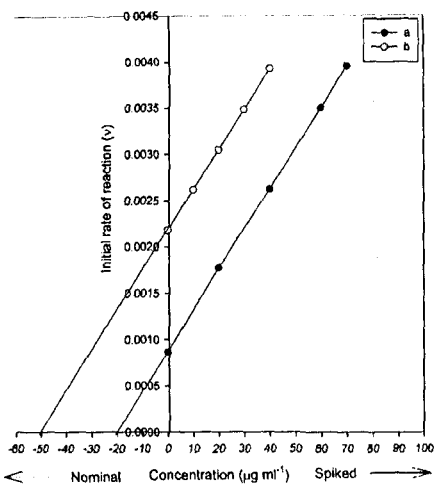

Fig. 2 Plot for the recovery evaluation of Pantec 20 through standard addition method: (a) 20 and (b) 50 μg ml^{-1}.

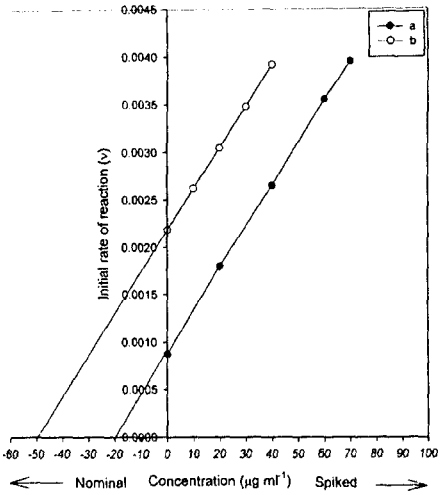

Fig. 3 Plot for the recovery evaluation of Pantodac 20 through standard addition: (a) 20 and (b) 50 μg ml^{-1}.

Table 5 Test of accuracy for analysis of pantoprazole in drug formulations by standard addition method

Formulation	Concentration/μg ml^{-1}			Coefficients of linear regression equation of standard addition			Recovery, %
	Theoretical	Spiked	Nominal	Intercept	Slope	r^a	
Pantec 20	20.0	0, 20, 40, 60, 70	20.004	8.766×10^{-4}	4.383×10^{-5}	0.9999	100.02
(Concept)	50.0	0, 10, 20, 30, 40	50.242	2.180×10^{-3}	4.339×10^{-5}	0.9994	100.48
Pantodac 20	20.0	0, 20, 40, 60, 70	20.357	8.729×10^{-4}	4.365×10^{-5}	0.9998	99.99
(Zydus Cadila)	50.0	0, 10, 20, 30, 40	50.233	2.150×10^{-3}	4.280×10^{-5}	0.9998	100.46

a. Coefficient of correlation.

Table 6 Analysis of pantoprazole in commercial dosage forms by proposed method and reference method at 95% confidence level

Formulation	Proposed method		Reference method		Paired t-value[b]	F-value[b]	θ_L[c]	θ_C[c]
	Rec., %	RSD, %[a]	Rec., %	RSD, %[a]				
Pantec 20 (Concept)	99.99	0.10	100.05	0.10	0.114	1.038	0.989	1.009
Pantodac 20 (Zydus Cadila)	100.06	0.10	99.98	0.10	0.279	1.201	0.991	1.011

a. Mean for 5 independent analyses. b. Theoretical t-value (ν = 8) and F-value (ν = 4, 4) at 95% confidence level are 2.306 and 6.39, respectively. c. In pharmaceutical analysis, a bias, based on recovery experiments, of ±2% is acceptable.

occurrence in pharmaceutical preparations.

Applicability of the proposed kinetic method

The proposed kinetic method has been successfully applied to the determination of pantoprazole in pharmaceutical preparations. The results obtained by the proposed method were compared to those of the reference method[11] using point and interval hypothesis tests. The results (Table 6) show that the Student's t- and F-values at 95% confidence level are less than the theoretical values, which confirmed that there is no significant difference between the performance of the proposed kinetic method and the reference method. The interval hypothesis test has also confirmed that no significant difference exists between the performances of the methods compared, as the true bias of all drug samples is < ±2.0%.

The performance of the proposed kinetic spectrophotometric method was compared with other existing conventional spectrophotometric methods (Table 7). It is apparent from Table 7 that the present procedure requires 4 min only for obtaining the initial rate of reaction, which is proportional to the concentration of pantoprazole. The reaction of pantoprazole with ammonium ferric sulfate and potassium ferricyanide requires 30 min to develop a stable color, which is mandatory for conventional spectrophotometric methods. Thus, the time of analysis is reduced when the kinetic spectrophotometric procedure was applied. The other conventional spectrophotometric methods using $FeCl_3$[10] and eosin and copper[11] need longer times to record the absorbance, whereas the method utilizing 2,3-dichloro-5,6-dicyano-1,4-benzoquinone[11] gives a stable color instantaneously. The drawback of these methods is that they employ an organic solvent while the proposed kinetic method is carried in an aqueous system.

Table 7 Comparison of the proposed kinetic spectrophotometric method with existing conventional spectrophotometric methods for the assay of pantoprazole in pharmaceutical formulations

Reagent	λ_{max}/ nm	Reaction time	Linear dynamic range/μg ml^{-1}	RSD, %	Ref.
$FeCl_3$ in ethanol	455	30 min at 60°C	30 - 300	1.52	10
2,3-Dichloro-5,6-dicyano-1,4-benzoquinone in acetonitrile	457	Immediately at 25°C	10 - 60	0.53	11
Iodine in chloroform	359	5 min at 25°C	17.7 - 141.6	1.21	11
Copper and eosin in chloroform	549	25 min at 70°C	4.3 - 25.9	0.81	11
Potassium ferricyanide and ammonium ferric sulfate	725	4 min at 35°C	5 - 90	0.78	This work

Conclusions

The proposed kinetic method is a selective one as the drug contains sulfoxide group, which preferentially reduces Fe(III) to Fe(II); subsequently Fe(II) reacts with potassium ferricyanide to form Prussian blue. In human and animals, pantoprazole is metabolized to pantoprazole sulfone, which did not give a positive result with the reagents used. This is a remarkable advantage of the method that Fe(III) in the presence of potassium ferricyanide selectively reacts with pantoprazole and give Prussian blue product. Point and interval hypothesis tests clearly proved that the proposed method has acceptable recovery with a bias of less than ±2%. The method is also useful due to its wide linear dynamic range (5.0 - 90.0 μg ml^{-1}) and high tolerance limit for common excipients found in drug formulations. Hence, these advantages encourage the application of the proposed kinetic method in routine quality control analysis of pantoprazole in industries, research laboratories and hospitals.

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